Pancreatic Islet Renin-angiotensin System: Its Role in Insulin Secretion and in Islet Transplantation

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Abstract

Background. The renin-angiotensin system (RAS) has long been known for its haemodynamic regulation by means of two major angiotensin II (Ang II) receptors, AT_1 and AT_2. A local angiotensin-generating system has been found in the exocrine pancreas. This study aimed, primarily, to investigate the existence of a local RAS in the pancreatic islets and, secondly, to elucidate its role in regulating insulin secretion and biosynthesis in the conditions of islet transplantation and type 2 diabetes. On the other hand, effects of AT_1 receptor blocker, losartan, on insulin release in islet graft and in type 2 diabetes were also investigated.

Methods. Real-time RT-PCR and Western blot were used to investigate if RAS components are present in the mouse pancreatic islets, which are subject to regulation by islet transplantation and diabetes. The localization of AT_1-receptors in islets was investigated by immunocytochemistry. Batch-type incubations of isolated islets from normal and diabetic mice were applied for studying the influence of Ang II and/or losartan on the glucose-stimulated insulin release, glucose oxidation and (pro)insulin and total protein biosynthesis. Perfusion experiments with whole pancreas were utilized to assess the effect of losartan on islet graft insulin release.

Results. Major components, namely angiotensinogen, ACE, AT_1- and AT_2- receptors,

were expressed in endogenous islets. AT_1-receptors were localized to pancreatic β cells. Exposure of the isolated islets to Ang II induced a dose-dependent inhibition of glucose-stimulated insulin release and inhibited (pro)insulin biosynthesis. This inhibitory action was fully preventable by pretreatment of the islets with losartan, an AT_1-receptor antagonist. Notably, a markedly increased expression of mRNA for the AT_1-receptor was observed in islets retrieved from islet transplants, a finding that was confirmed at the protein level. Graft perfusion experiments showed a markedly improved first phase of insulin release to glucose stimulation in transplanted islets when exposed to losartan. On the other hand, mRNA for AT_1, AT_2 receptors and angiotensinogen were upregulated by type 2 diabetes in the pancreas. A decrease in insulin-secreting β cell number was found in diabetic mice islets. Losartan was found to increase insulin release in type 2 diabetes.

Conclusion/interpretation. These data indicate the existence of an islet RAS. This system provides an inhibitory role for locally produced Ang II of glucose-stimulated insulin secretion, an effect mediated by the AT_1 receptors located on the surface of the islet β cells. The beneficial effect of losartan on insulin release in islet transplantation and type 2 diabetes may provide a new insight into the treatment of diabetes mellitus.

摘要

背景 — 腎素-血管緊張素系統以它在血流動力學中的調節作用而知名。 它對血流的調節是通過兩個主要的血管緊張素 II(Ang II)受體，受體一型和受體二型而達到的。 早期研究在胰線的外分泌腺中發現了內在的腎素-血管緊張素系統。目前的研究旨在查出胰島細胞上是否也存在着一個內在的腎素-血管緊張素系統，而此內在系統很可能在胰島移植術和 2 型糖尿病中扮演着調節胰島素分泌的重要角色。 此外，此項研究也對血管緊張素 II 受體一型對抗劑(losartan)在胰島移植術和 2 型糖尿病中，對胰島素分泌所引起的作用作出了調查。

方法 — 我們利用了即時反轉錄酶—聚合酶鏈反應和西方墨點法來檢測胰島細胞是否附有腎素-血管緊張素系統的成員及其在胰島移植術和 2 型糖尿病中的調節作用；同時也運用了免疫細胞化學技術來測定血管緊張素 II 受體一型在胰島上的位置。 另外，我們分批温育從胰線分離出來的胰島，並探索胰島上的血管緊張素 II 在受葡萄糖刺激下的胰島素分泌、葡萄糖氧化、胰島素原和總蛋白質合成中所起到的作用。 除此以外，還利用了胰腺灌注實驗調查血管緊張素 II 受體一型對抗劑在胰島移植物中調節胰島素分泌之作用。

結果 — 腎素-血管緊張素系統的主要成員，包括血管緊張素原、血管緊張素轉換酶、血管緊張素 II 受體一型和二型均在內生的胰島上表達。 其中的血管緊張

素 II 受體一型局限在胰島乙細胞上。 在分離的胰島中，血管緊張素 II 對於葡萄糖刺激的胰島素分泌和胰島素原合成起到了劑量依賴性的抑制作用；而此抑制效用是可利用血管緊張素 II 受體一型對抗劑來預防的。 另外，血管緊張素 II 受體一型的信息核糖核酸和蛋白質在胰島移植物中都有顯著的調升。 胰島移植物灌注實驗顯示，血管緊張素 II 受體一型對抗劑在移植的胰島中能有效地提高受葡萄糖刺激下的一期胰島素分泌。 另一方面，在 2 型糖尿病的情況下，血管緊張素原、血管緊張素 II 受體一型和二型的信息核糖核酸也有上調；而分泌胰島素的胰島乙細胞數量則有顯著減少。 此外，2 型糖尿病的情況下的胰島素分泌也能通過使用血管緊張素 II 受體一型對抗劑而得到提高。

結論 — 從實驗數據中我們可以看到，胰島上的確存在着內在的腎素-血管緊張素系統。 通過胰島乙細胞上的血管緊張素 II 受體一型的轉達，此內在系統生成的血管緊張素 II 對受葡萄糖刺激下的胰島素分泌能引起抑制作用。 另外，數據顯示血管緊張素 II 受體一型對抗劑有利於胰島移植術和 2 型糖尿病中的胰島素分泌，此項研究結果將爲糖尿病的治療帶來新的曙光。

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List of Abbreviations

Angiotensin converting enzyme	ACE
Angiotensin converting enzyme-related carboxypeptidase 2	ACE-2
Angiotensin converting enzyme inhibitor	ACEI
Angiotensinogen	Ao
Angiotensin I	Ang I
Angiotensin II	Ang II
Angiotensin III	Ang III
Angiotensin IV	Ang IV
Angiotensin II receptor type 1	AT_1 receptor
Angiotensin II receptor type 2	AT_2 receptor
Angiotensin II receptor blockers	ARBs
Bovine serum albumin	BSA
Captopril Primary Prevention Project	CAPPP
Cholecystokinin	CCK
End stage renal disease	ESRD
Gastrointestinal	GI
Glucose transporter-2	Glut-2
Heart Outcomes Prevention Evaluation trial	HOPE
Impaired glucose tolerance	IGT
Islet amyloid polypeptide	IAPP
Krebs-Ringer bicarbonate buffer	KRBB
Losartan Intervention For Endpoint Reduction in Hypertension study	LIFE
Nateglinide and Valsartan in Impaired Glucose Tolerance Outcomes Research	NAVIGATOR
Oral glucose tolerance test	OGTT
Reduction in Endpoints in Non-Insulin-Dependent Diabetes Mellitus with the Ang II Antagonist Losartan	RENAAL
Renin-angiotensin system	RAS
Reverse transcriptase polymerase chain reaction	RT-PCR
Streptozotocin	STZ
Transforming growth factor- β	TGF-β
Type 1 diabetes	T1DM
Type 2 diabetes	T2DM

Chapter 1 Introduction

1.1 Pancreas and its functions

1.1.1 Structure of pancreas

The pancreas is a flat and elongated gland, analogous in its structure to the salivary glands, though softer and less compactly arranged than that organ (Fig.1-1A). It is long and irregularly prismatic in shape; its right extremity, being broad, is called the head, and is connected to the main portion of the organ, or body, by a slight constriction, the neck; while its left extremity gradually tapers to form the tail. The head is embedded in the curvature of the duodenum while the tail is tucked into the spleen. It is situated transversely across the posterior wall of the abdomen, at the back of the epigastric and left hypochondriac regions [Gray 1918].

The bulk of the pancreas is composed of pancreatic exocrine cells and their associated ducts. Pancreatic exocrine cells are arranged in grape-like clusters called acini (Fig.1-1B). The exocrine cells themselves are packed with membrane-bound secretory granules which contain digestive enzymes that are exocytosed into the lumen of the acinus. From there these secretions flow into larger and larger,

intralobular ducts, which eventually coalesce into the main pancreatic duct which drains directly into the duodenum.

Embedded within this exocrine tissue are roughly one million small clusters of cells called the Islets of Langerhans (Fig.1-1B), which are the endocrine cells of the pancreas and secrete insulin, glucagon, somatostatin, pancreatic polypeptides and several other hormones.

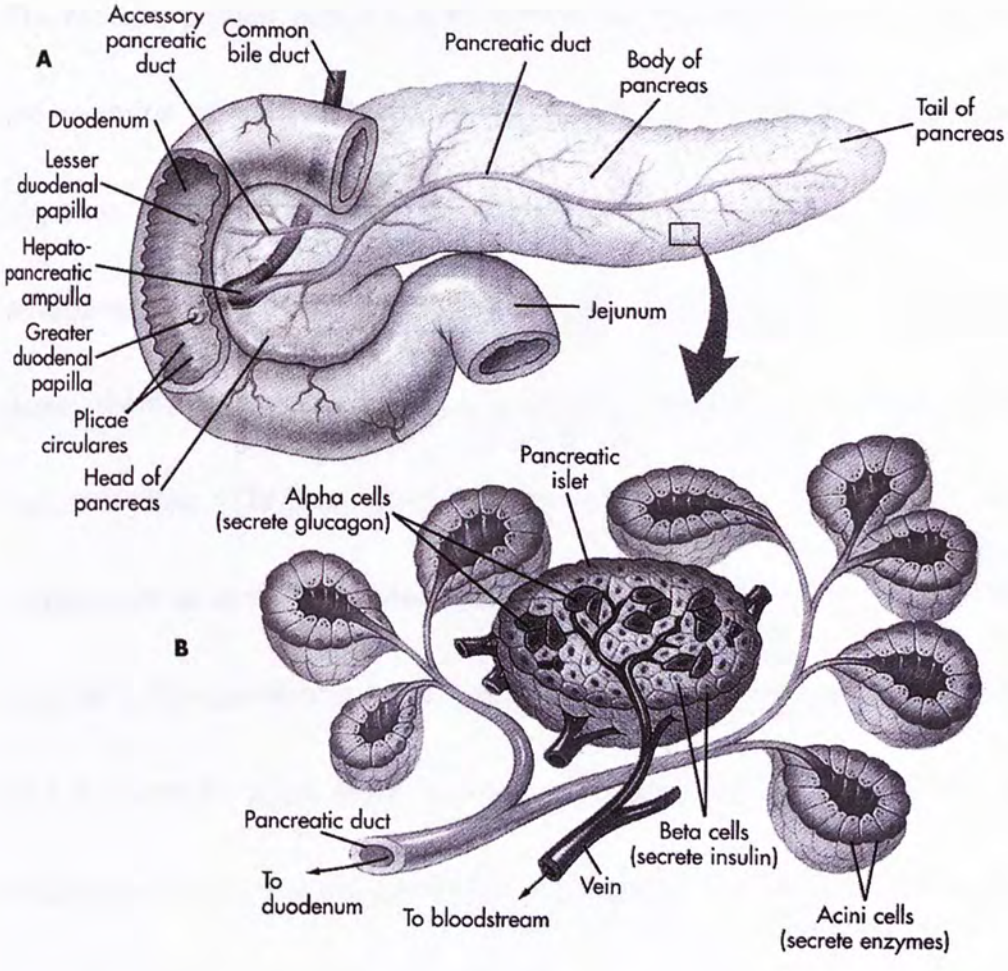


Fig 1-1. Structure of pancreas

A. Overview of the pancreas. B. Functional units of the pancreas.

Figure is adopted from [Piano and Huether 1997]

1.1.2 Exocrine function

The exocrine portion, comprising 85 % of the mass of the pancreas, is responsible for the secretion of fluid and various enzymes which are involved in the process of digestion. Pancreatic acinar cells synthesize and secrete at least 10 different hydrolases, including α-amylase, lipase, proteases, ribonuclease and deoxyribonuclease, which are the major enzymes responsible for luminal digestion of macronutrients. Trypsin inhibitor, a protein, is also synthesized and secreted by acinar cells as partial protection against early activation of trypsinogen and other proteases, thus preventing autodigestion. Colipase, another precursor protein, acts as a co-factor for lipase in fat digestion. The major dietary carbohydrate, starch, is hydrolyzed by the actively secreted α-amylase, and is converted into trisaccharides, maltose and α-limit dextrins. The pancreatic lipase breaks down the major dietary fat, triglyceride, into 2-monoglyceride and free fatty acids. The ribonuclease and deoxyribonuclease are also secreted in active forms and they are responsible for breaking down nucleic acids into nucleotides. Sufficiency of pancreatic exocrine secretion is essential for efficient digestion of food, and the subsequent normal absorption [Singer 1993].

Secretion from the exocrine pancreas is regulated by both neural and hormonal controls (Chey & Chang 2001). Like the stomach, the pancreas is innervated by the vagus nerve, which applies a low level of stimulus to secretion in response to anticipation of a meal. However, the most important stimuli for pancreatic secretion come from three hormones secreted by the enteric endocrine system. Cholecystokinin (CCK) is synthesized and secreted by enteric endocrine cells (I cells) located in the duodenum. Its secretion is strongly stimulated by the presence of partially digested proteins and fats in the small intestine. As gastric chyme floods into the small intestine, CCK is released into blood and binds to receptors on pancreatic acinar cells, rendering them to secrete large quantities of digestive enzymes. Secretin is also a product of enteric endocrine cells located in the epithelium of the proximal small intestine. Secretin is secreted in response to acid in the duodenum when acid-laden chyme from the stomach is emptied into the duodenum. The predominant effect of secretin on the pancreas is to stimulate pancreatic duct cells to secrete water and bicarbonate for the neutralization of the pH of the pancreatic juice. As soon as this occurs, the enyzmes secreted by the acinar cells are flushed out of the pancreas through the pancreatic duct into the duodenum. Gastrin, with a structural similarity to CCK, is secreted in large amounts by the

stomach in response to food stimuli. In addition to regulating gastric acid secretion by the parietal cell, gastrin stimulates pancreatic acinar cells to secrete digestive enzymes.

Besides the classical neuronal and hormonal controls, many regulatory factors such as growth factors, prostaglandin and vasoactive peptides are found to stimulate exocrine pancreatic secretion of bicarbonate and digestive enzymes. In this regard, the vasoactive peptide angiotensin II (Ang II) has been demonstrated to play a role in stimulating the α-amylase secretion in the pancreatic acinar cells [Tsang *et al*. 2004]. The pancreatic endocrine hormones such as glucagon, somatostatin and pancreatic polypeptide are factors for inhibiting the exocrine secretion of pancreas. The vital role of exocrine pancreas is manifested with the fact that the insufficiency in pancreatic exocrine secretion as occurred in pancreatitis leads to maldigestion and malabsorption of our body.

1.1.3 Endocrine function

1.1.3.1 Pancreatic islet and islet cells

The endocrine pancreas represents an anatomically small part of the pancreas. It secretes hormones that are important regulators of energy metabolism and fuel homeostasis in the body. The endocrine pancreas consists of groups of cells known as the islets of Langerhans, which are imbedded in the exocrine portion of the gland. Islets comprise only about 1 % to 2 % of the total mass of the pancreas, although the average human pancreas has about one to two million islets. Each islet is richly supplied with blood vessels, and the hormones that islet cells secrete enter these blood vessels directly. Islets are composed of four major cell types, as listed in Table 1.1. In rodents, each islet is composed of 2000-4000 cells of which 60-70 % are β cells, 10 % are somatostatin-producing δ cells and 20-30 % are either glucagon-producing α cells or pancreatic polypeptide-producing F cells. This depends on whether the islets are located in the head, body or tail of the pancreas. Each cell type synthesizes and secretes different hormone. Glucagon and insulin, which are produced in the α cells and β cells, respectively, are important hormones involved in the regulation of blood glucose homeostasis. β cells also secrete islet

amyloid polypeptide (IAPP). This is a 37-amino-acid peptide originally derived from islet amyloid deposits in pancreas material form patients with long-standing non-insulin dependent diabetes or insulinomas [Karlsson *et al.* 1998]. Somatostatin produced by δ cells regulates hormonal secretion from α and β cells. The remaining F cells produce pancreatic polypeptide and its roles have not yet been fully investigated. Each individual islet shows an orderly arrangement of the different cell types. Insulin secreting β cells tend to be centrally located and are generally the most numerous (see Table 1-1). The less numerous glucagons-secreting α cells are located peripherally in the islet. The δ cells that produce somatostatin are scattered over between α cells and β cells. As somatostatin inhibits hormonal secretion from both α cells and β cells, this arrangement of cells may be important with regard to paracrine actions of somatostatin. F cells show roughly the same distribution as the δ cells, although they are considerably fewer in number. Islets also receive innervation from both the sympathetic and parasympathetic divisions of the autonomic nervous system. Parasympathetic stimulation usually augments islet hormonal secretion while sympathetic stimulation can have either a stimulatory or inhibitory effect, depending on whether β-adrenergic or α-adrenergic stimulation dominates.

Table 1-1. Major cell types of the Islets of Langerhans and the hormones they produced. The percent of each cell type in the islets is also shown.

Name	Hormone produced	% of total islet
α cell	Glucagon	25
β cell	Insulin Islet Amyloid Polypeptide (IAPP)	60
δ cell	Somatostatin	10
F cell	Pancreatic polypeptide	1

The remaining 4% consists of connective tissue and blood vessels.

1.1.3.2 Regulation of insulin secretion

Insulin, secreted by β cells, is a peptide hormone that consists of an A-chain (21 amino acids) and a B-chain (30 amino acids) held together by two disulfide (S-S) bonds. It is derived from a larger precursor molecule known as proinsulin. Proinsulin is first synthesized in the rough endoplasmic reticulum of β cells. Proinsulin consists of a single peptide chain that contains both the A and B chains of insulin linked by a third connecting peptide segment. As the hormone is packaged into secretory vesicles within the β cells, proinsulin is converted into insulin by proteolytic enzymes that clip the peptide chain in two places. The peptide segment that is removed is known as C-peptide. When insulin is secreted into the blood, an equal amount of C-peptide is also secreted. C-peptide does not appear to have any established biological secretion. However, because it is secreted in a 1:1 molar ratio with insulin, it is a useful marker for insulin secretion. Most of the insulin that is secreted into the portal blood is removed in a first pass through the liver. In contrast, C-peptide is not extracted by the liver at all. As a result, whereas measurements of the insulin concentration in systemic blood do not quantitatively mimic the secretion of insulin, measurements of C-peptide do. C-peptide is eventually excreted in the urine.

In the broadest sense, insulin secretion is governed by a feedback relationship with exogenous nutrient supply. When substrate supply is abundant, insulin is secreted accordingly. Insulin then stimulates use of these incoming nutrients and simultaneously inhibits the mobilization of analogous endogenous substrates. When nutrient supply is low or absent, insulin secretion is dampened and mobilization of endogenous fuels is enhanced. Several factors promote insulin secretion, including increased blood glucose, amino acids, fatty acids, gastrointestinal hormones, neural and pharmacological stimuli, and other hormones secreted by the pancreas. Glucose is the primary stimulus of insulin secretion. Because insulin in turn stimulates the use of glucose, this substrate-hormone pair forms a feedback system for close regulation of plasma glucose levels. The relationship between plasma insulin and plasma glucose is sigmoidal. Several other factors influence insulin secretion, although none is as physiologically important as glucose. Table 1-2 summarizes the major physiological stimulatory and inhibitory regulators of insulin release. When glucose is given orally, a greater insulin response is elicited than when plasma glucose is comparably elevated by intravenous administration. This augmented insulin response to oral glucose is accountable for by one or more intestinal hormones that are released in response to meals. They are

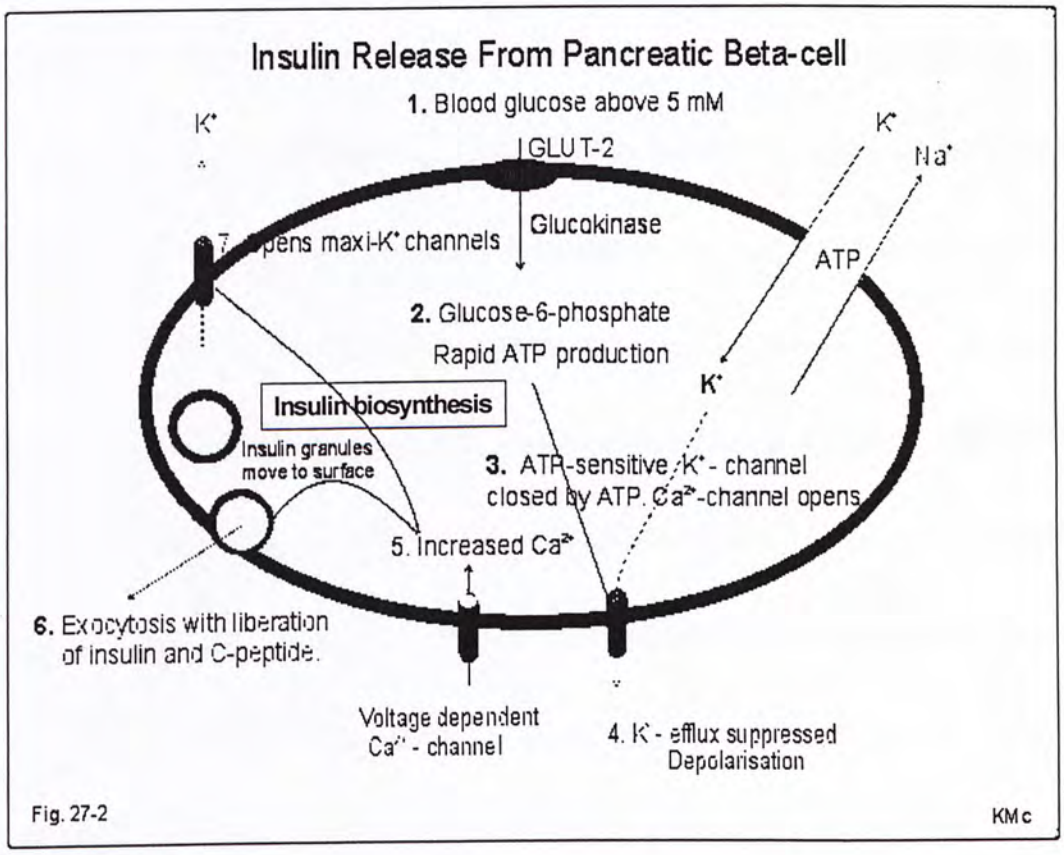
called incretins which are capable of potentiating glucose-stimulated insulin secretion. Glucagon-like peptide 1 and gastric inhibitory polypeptide are most important of these insulinogogues. This prompt gastrointestinal mechanism of insulinogenesis moderates the early rise in plasma glucose that follows the ingestion and absorption of a carbohydrate meal. Several amino acids, especially arginine, are known to stimulate insulin secretion. Glucose and amino acids are synergistic stimulators of insulin release, so that the plasma insulin rise that follows a meal represents more than the additive effect of its carbohydrate and protein content. In contrast, somatostatin released within pancreatic islets (paracrine or neurocrine) and from intestinal cells (endocrine) may inhibit insulin responses to meals.

Table 1-2. Major physiological stimulatory and inhibitory regulators of insulin release

Increased by	Decreased by
D-Glucose	Fasting
Galactose	Exercise
Mannose	Endurance training
Glyceraldehyde	Somatostatin
Protein	Galanin
Arginine	Pancreastatin
Lysine	Leptin
Leucine	Interleukin-1
Alanine	α-Adrenergic activity
Ketoacids	Prostaglandin E_2
Free fatty acids	Diazoxide
Potassium	
Calcium	
Glucagon	
Glucagon-like peptide 1	
Gastric inhibitory polypeptide	
Secretin	
Cholecystokinin	
Vagal activity	
Acetylcholine	
β-Adrenergic activity	
Sulfonylurea drugs	
Meglitinides	

1.1.3.3 Mechanism for glucose-stimulated insulin release

When plasma glucose level is increased, a specific glucose transporter (Glut-2), concentrated in the microvilli between β cells, facilitates diffusion of glucose into the β cell. This helps maintain the glucose concentration in the β cell at a level that is essentially equal to that of the interstitial fluid. Phosphorylation of glucose by the enzyme glucokinase then generates an insulin-releasing signal downstream from glucose-6-phosphate. At the same time that glucose is being oxidized, there is a rapid increase in intracellular ATP concentration, i.e. ATP/ADP increase occurs in the β cell. An ATP-sensitive K^+ channel closes, K^+ efflux from the β cell is suppressed, and the cell depolarizes. Depolarization opens a voltage-regulated Ca^{++} channel, and the concentration of intracellular Ca^{++} rapidly increases. The elevated Ca^{++} concentration activates the mechanism for secretory granule movement along the microtubules. A monomeric G protein attached to the secretory vesicle interacts with special plasma membrane proteins (fusins). This interaction leads to the fusion of the granule with the membrane. Exocytosis of insulin follows. Fig. 1-2 shows a schematic diagram in the regulation of insulin release.



http://arbl.cvmbs.colostate.edu/hbooks/pathphys/endocrine/pancreas/insulin.html

Fig. 1-2. Schematic diagram of regulation of insulin secretion by β cells:(1) GLUT-2 facilitates diffusion of glucose into β cells. (2) Glucokinase catalyzes phosphorylation and raise glucose-6-phosphate levels, subsequently leads to increased ATP levels. (3) Increase in ATP levels closes a potassium channel and opens a calcium channel. (4) Potassium efflux from the β cells is suppressed and the cell depolarized. (5) Voltage dependent calcium channel opened by depolarization. (6) Increased calcium activated exocytosis of insulin.

1.1.3.4 Bi-phase response of insulin secretion

Both *in vitro* and *in vivo*, insulin secretion exhibits a biphasic response to a continuous glucose stimulus (Fig. 1-3). Within seconds of exposure to glucose, an immediate pulse of insulin is released that peaks at 1 minute and then returns toward baseline. After 10 minutes of continuous stimulation, a second phase of secretion begins. During this phase, insulin plasma levels rise more slowly and reach a second plateau, which can be maintained for many hours in normal individuals. The initial increase is due to secretion of preformed insulin, which is rapidly depleted. The second rise in insulin reflects a considerable amount of newly synthesized insulin that is released subsequently. Clearly, elevated glucose not only simulates insulin secretion, but also transcription of the insulin gene and translation of its mRNA. The biphasic response may result from (1) rapid insulin generation followed by the slow removal of a substance that is formed after glucose stimulation and acts as a feedback inhibitor of insulin release, (2) granules with different sensitivities to glucose, and (3) glucose stimulation of insulin synthesis that sustains the later secretory phase.

A normal biphasic response curve can be obtained by performing oral glucose tolerance test (OGTT) on human subjects or experimental animals. OGTT measures the body's ability to use glucose. To conduct an OGTT test, a person has to drink 75 grams of glucose after a 12-hour overnight fast. Blood samples are drawn at various times and the concentration of glucose is determined. It is well know that the administration route of glucose influences the amount of insulin secreted by the pancreas. If glucose is given orally, a greater increase in insulin secretion occurs as compared to when the same amount of glucose is given intravenously. This difference is due to a stimulatory effect of incretins on insulin secretion. Soon after food enters the Gastrointestinal (GI) tract, the blood glucose concentration begins to rise.

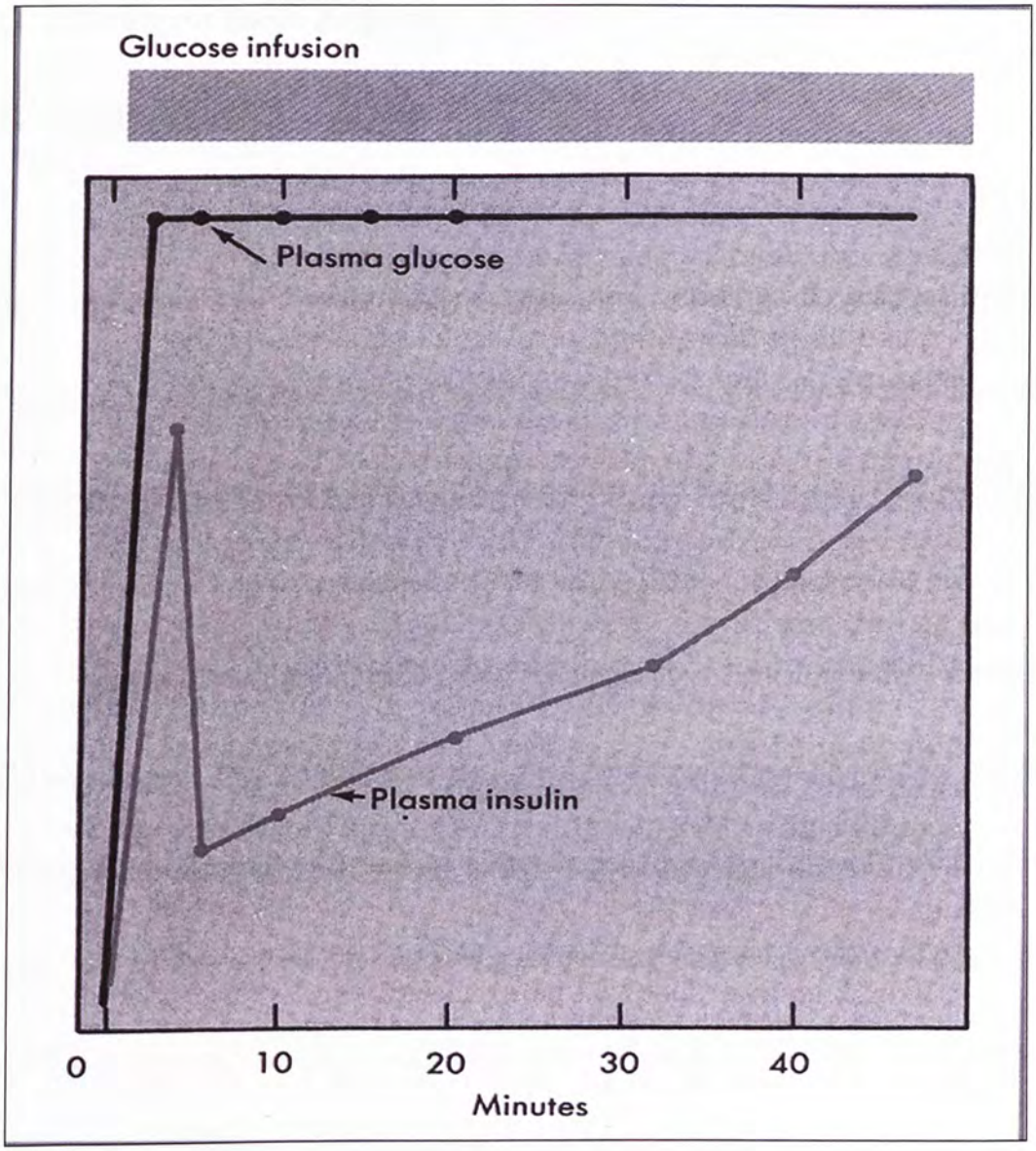


Fig. 1-3. Biphasic response of insulin secretion.
Figure is adopted from [Genuth 2003]

1.2 Pancreatic Renin-Angiotensin system

1.2.1 Circulating RAS and local RAS

The renin-angiotensin system (RAS) is critically involved in the development and maintenance of cardiovascular and renal diseases. Such a blood-borne hormonal system plays an important role in the hormonal mechanisms that regulate blood pressure and electrolyte homeostasis [Reid *et a.l* 1978]. The classical circulating RAS cascade contains several key components, including the precursor angiotensinogen, two critical enzymes renin and angiotensin-converting enzyme (ACE), angiotensin I (Ang I), and the bioactive peptides Ang II, Ang III, Ang IV and Ang (1-7), as well as multiple receptor subtypes including AT_{1a}, AT_{1b}, AT_2, AT_4 and AT_7 [Gasparo *et al.* 2000]. The major enzyme of the RAS is renin, which belongs to the family of aspartyl proteases and which shows a high specific affinity for its natural substrate, angiotensinogen. Renin is produced by the juxtaglomerular cells in the kidney and is secreted into the circulation. The major substrate of renin is the glycoprotein, angiotensinogen. Plasma angiotensinogen is produced by the liver. Renin cleaves angiotensinogen to form a decapeptide, which is called Ang I. Ang I is itself an inactive peptide and it is converted to Ang II by the action of endothelium

derived ACE. ACE is a dipeptidyl carboxypeptidase that converts angiotensin I to the octapeptide, called Ang II, a potent vasoconstrictor. In addition, ACE is responsible for the degradation of bradykinin – a potent vasodilator. In this regard, ACE inhibitor can lead to an accumulation of bradykinin, thus causing vasodilation and elevated vascular permeability. ACE is present in nearly all human tissues and body fluids, and is found in highest quantities in the vascular endothelium of the lungs and peripheral tissues. All major actions of Ang II are mediated by binding to receptors on the cell surface that are highly specific for Ang II. Today it is known that most, if not all, of the effects of Ang II are mediated via the Ang II type 1 receptor (AT_1). This receptor subtype occurs throughout the body in all species and it mediates most of the well-established physiological and pathophysiological actions of Ang II [Timmermans *et al.* 1993]. Moreover, Ang II could be further converted into a number of bioactive metabolites such as Ang III and Ang IV by various aminopeptidases. Another angiotensin metabolite, Ang 1-7 could also be generated upon the action of ACE-2, a homologue of ACE that exerts a cleavage of Pro-Phe bond from Ang I [Corvol *et al.* 1995]. In addition to the dominant role of Ang II, emerging evidence has shown that Ang III, Ang IV and ang 1-7 also exhibit either complimentary or contrasting biological properties via the mediation of their

respective receptors [Ardaillou and Chansel 1997]. Fig. 1-3 shows a schematic diagram of the synthesis of bioactive angiotensin peptides.

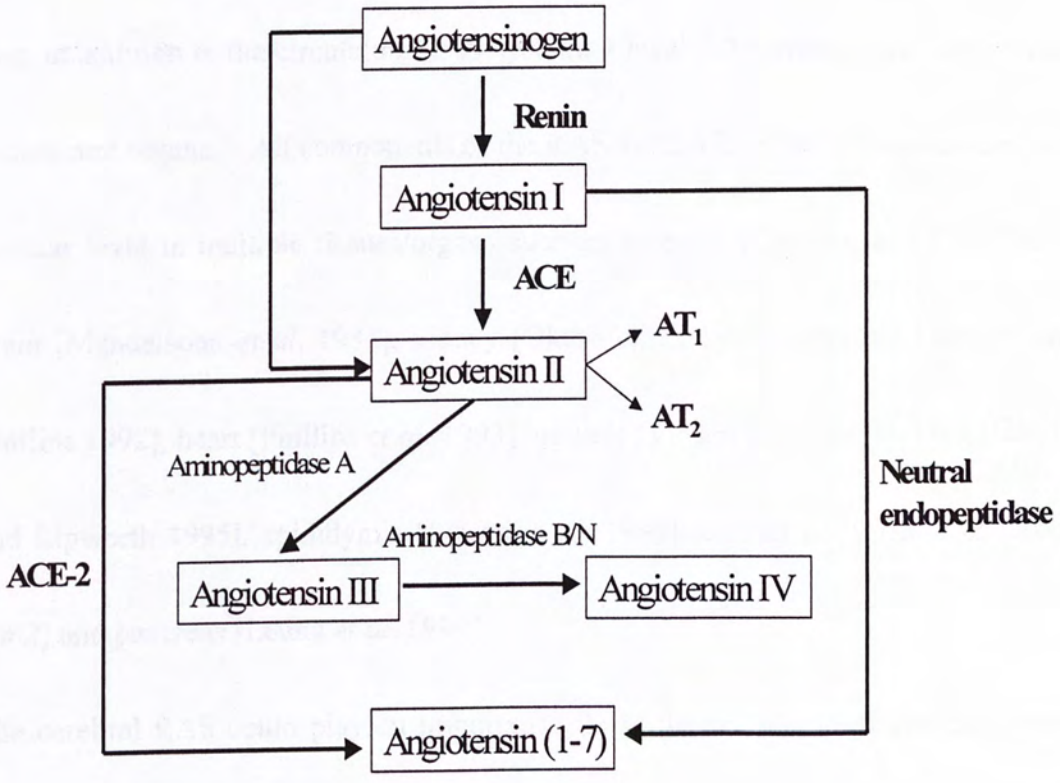


Fig. 1-3. A schematic diagram depicting the synthesis of bioactive angiotensin peptides using renin, ACE and alternate enzymes. Figure is adopted from P.S. Leung (Current protein and peptide science, 2004).

It was originally thought that the RAS was an endocrine system, present only in the circulation. However, molecular biological research in the last decade has shown that, in addition to the circulating RAS system, a local RAS system exists in various tissues and organs. All components of the RAS system have been demonstrated at a cellular level in multiple tissues/organs such as adrenals [Capponi and Catt 1980], brain [Mendelsohn *et al.* 1984], kidney [Okura *et al.* 1992], pituitary [Trolliet and Phillips 1992], heart [Phillips *et al.* 1993], gonads [Vinson *et al.* 1997], lung [Cargill and Lipworth 1995], epididymis [Leung *et al.* 1999], carotid body [Lam & Leung 2002] and pancreas [Leung *et al.* 1999].

The cerebral RAS could play an important role in the regulation of cerebral blood flow during hypoxia in rabbit as the increase in cerebral blood flow was attenuated by RAS inhibitors [Mazen *et al.* 1995]. The adrenal RAS has been reported to regulate the tissue growth and functions of glomerulosa in rat [Vinson 1995]. Besides, the cardiac RAS has been shown to play a regulatory role in cardiac hypertrophy and remodeling [Dostal 2000] as well as in the pathogenesis of atherosclerosis [Shieffer *et al.* 2000]. In the epididymis, locally generated Ang II plays a paracrine/autocrine pathway in regulating anion and fluid secretion by the epididymis [Leung and Sernia 2003]. In the carotid body, the functional expression

of AT_1 receptor in the type-I cells of rat carotid body has been demonstrated [Fung *et al.* 2001]. Ang II in carotid body can mediate the intracellular calcium release and thus regulating the neural activity of the carotid body chemoreceptors [Leung *et al.* 2003]. A large body of work has substantiated that the locally generated Ang II may have significant proinflammatory actions, such as the production of reactive oxygen species, inflammatory cytokines, chemokines, and the modulating abilities in cell growth and injury of the end-tissue. In this regards, Ang II was reported to be responsible for the hepatic fibrogenesis [Paizis *et al.* 2001]. In addition, the pulmonary Ang II has been demonstrated to act as a potential profibrotic mediator in the lung via the activation of AT_1 receptor [Marshall *et al.* 2000].

Activation of the RAS produces short-term and long-term effects. The short-term effects (sodium and water retention, vasoconstriction, and positive inotropic effects on the heart) are probably produced by the circulating RAS system. In contrast, activation of the tissue RAS is likely to result in long-term effects such as hypertrophy of the heart and blood vessels [Ferario 1990]. To this end, a fuller understanding of local RAS could give rise to important clinical implications for a number of diseases.

1.2.2 RAS inhibitors

1.2.2.1 Angiotensin converting enzyme inhibitor

The fact that angiotensins contribute to the pathogenesis of several diseases stimulated the development of drugs that inhibit the RAS. This has been an exciting and fruitful area of research, particularly during the past decade, in which potent and specific inhibitors on the formation or actions of Ang II have been developed (Fig.1-4).

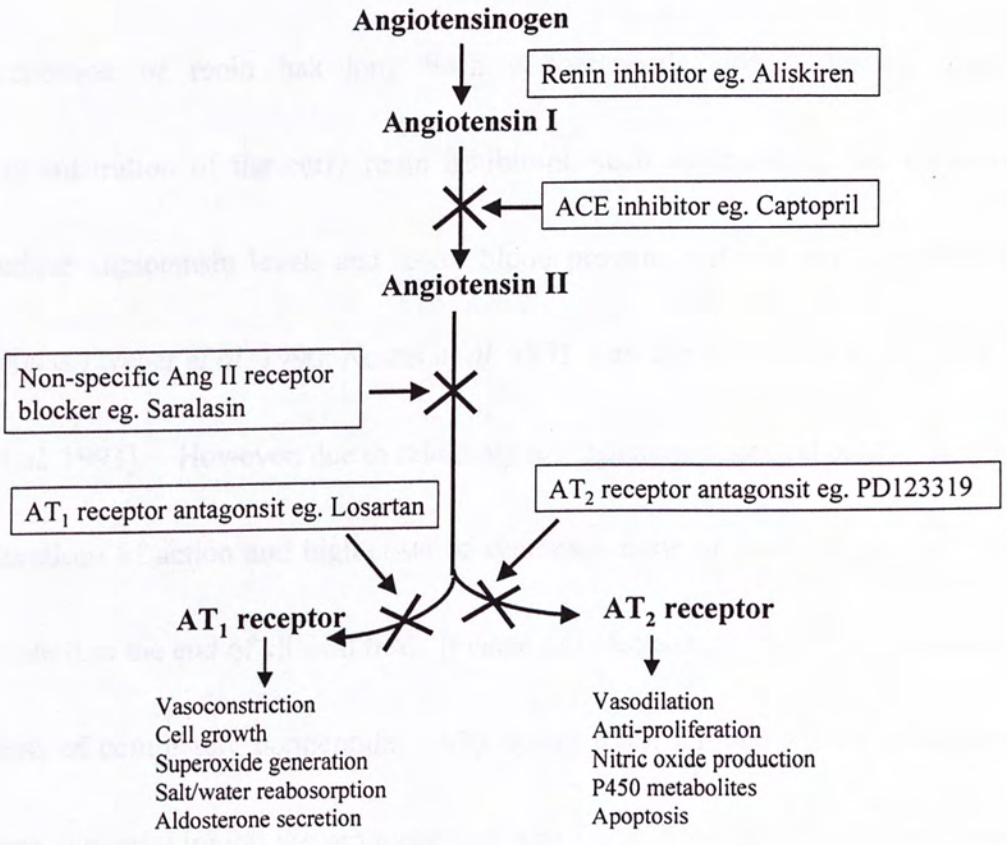


Figure 1-4 Schematic presentation of the RAS cascade with its relationship to ACE inhibitor and Ang II receptor antagonists.

As renin catalyzes the first and rate-limiting step of the system and has high specificity for angiotensinogen, blockade of the production of Ang II by direct inhibition of renin has long been a therapeutic goal. Indeed, intravenous administration of the early renin inhibitors, such as enalkiren and remikiren, did reduce angiotensin levels and lower blood pressure without any important adverse effects [Weber *et al.* 1990; Neutel *et al.* 1991; van den Meiracker *et al.* 1993; Kobrin *et al.* 1993]. However, due to relatively low potency, poor oral bioavailability, short durations of action and high costs of synthesis, none of these peptide inhibitors has made it to the end of clinical trials [Fisher and Hollenberg 2001]. Nowadays, a new class of completely nonpeptide, orally active renin inhibitors such as aliskiren, has been shown to inhibit the production of Ang I and II and to reduce blood pressure in sodium-depleted marmosets [Stanton *et al.* 2003].

The other two most widely used RAS inhibitors are ACE inhibitor and Ang II receptor antagonists. ACE is a key enzyme in the RAS for the conversion of the bioinactive Ang I into the effector peptide Ang II. The first ACE inhibitor Captopril was created in 1975 with the application of structure-based drug design technology [Pfeffer *et al.* 1988]. The available ACE inhibitors can be divided into 3 groups: the sulphydryl-containing group such as captopril, the carboxyl-containing group such as

enalapril and ramipril, and the phosphorous-containing group such as fosinopril [Wyvratt 1988; Voors *et al.* 1995]. Generally, ACE inhibitors belonging to the carboxyl group are more potent than captopril but carrying lower bioavaliabilities while the phosphorous-containing group has longer duration of effectiveness. They both reduce the formation of Ang II by blockade of ACE.

1.2.2.2 Non-specific Ang II receptor blocker

Since Ang II is the key active peptide of the RAS, Ang II receptor blockers are thought to be more effective than the action of ACE inhibitor. It blocks the RAS pathway more specifically at the last step, without worrying about the accumulation of bradykinin and the ACE-independent formation of Ang II. Saralasin, which is a peptide-based analog of Ang II, acts as an antagonist for Ang II by competitively blocking both the AT_1 and AT_2 receptors. Saralasin was found effective in treating hypertension in patients with high renin concentration, but not in patients with low plasma level of renin [Satia *et al.* 1995]. Given that AT_1 and AT_2 receptors may counteract to each other, the blockade of RAS by saralasin could only represent the compensated results of the two receptor subtypes. In view of this, selective receptors for AT_1 and AT_2 became more noteworthy.

1.2.2.3 Specific AT_1 receptor antagonist

The AT_1 receptor belongs to the seven transmembrane class of G-protein coupled receptor and is encoded by a 359-amino acid protein. Two isoforms, AT_{1a} and AT_{1b} have been identified in rodents [de Gasparo *et al.* 2002]. The specific AT_1 receptor antagonist interacts with amino acids in the transmembrane region of the AT_1 receptor and occupies space among the seven helixes, thus preventing the binding of Ang II. Losartan (Dupont pharmaceuticals, Dup 753; Merck and Co., MK954) is the first marketable non-peptide AT_1 receptor antagonist [Timmermans *et a*l. 1993]. It overcomes the drawbacks of saralasin, with higher bioavailability, selective antagonism to AT_1 receptor without exhibiting agonist effect in the course of the RAS blockade. The AT_1 receptor antagonist was approved by FDA in 1995 for the treatment of hypertension, either alone or in combination with other antihypertensive agents. Other specific AT_1 receptors including candesartan cilexetil, irebesartan and valsartan are biphenyl analogs of losartan. These antagonists for AT_1 receptors have different oral bioavailabilities, protein binding properties and acting duration time.

1.2.2.4 Specific AT_2 receptor antagonist

The binding activity of Ang II with AT_2 receptor is similar to that of AT_1 receptor. The AT_2 receptor also possesses a seven transmembrane domain but is encoded by a 363-amino acid protein [de Gasparo *et al.* 1995]. A series of 1,2,3,4-tetrahydrosioquinoline-3-carboxylic acids including EXP655, EXP801, PD121981, PD123317 and PD123319 have been developed with high binding affinities selectively to AT_2 receptor for Ang II. PD123319 is the most commonly used non-peptide AT_2 receptor antagonist.

1.2.3 RAS in the pancreas

1.2.3.1 Expression and localization of pancreatic RAS

In recent years, the existence of a local angiotensin-generating system in the pancreas of various species was shown. The expression of the bioactive peptides Ang II, Ang III and Ang (1-7), the precursor angiotensinogen, as well as the AT_1 and AT_2 receptor subtypes were first demonstrated within the dog pancreas [Chappel *et al.* 1991; 1992]. Subsequent studies demonstrated the presence of key RAS components in rat [Ghiani and Masinin 1995; Leung *et al.* 1999], mouse [Leung *at al.* 1998] and human

[Tahmasebi *et al.* 1999]. In terms of the acinar cells in pancreas, mRNA expression of all components of the RAS including AT_2, AT_{1a} and AT_{1b} subtypes, as well as renin, angiotensinogen and ACE were demonstrated in the AR42J cell line [Chappell *et al.* 2001]. With autoradiography, Ang II receptors were shown to be distributed throughout the pancreas, but with the highest density on acinar cells. In rat pancreas, Ang II receptors are mainly located in islets, and preferentially to the surface of α and δ cells [Ghiani and Masini 1995]. The majority of binding sites in the canine pancreas are AT_2 receptors, although AT_1 receptors can also be found [Chappell *et al.* 1992]. In rodents AT_1 and AT_2 receptors were specifically localized to various elements of the pancreas [Leung *et al.* 1997]. Major RAS components at gene and protein levels were unequivocally expressed in the rat pancreas [Leung *et al.* 1999]. In the human pancreas, the presence of both AT_1 receptors and renin have been demonstrated in islets [Tahmasebi *et al.* 1999]. All these results substantiate the existence of a pancreatic RAS, which have important roles in the regulation of pancreatic exocrine and endocrine functions [Leung and Carlsson 2001].

1.2.3.2 Regulation of pancreatic RAS and its clinical relevance

The pancreatic RAS is subjected to various physiological and pathophysiological regulations. In terms of endocrine pancreas, the local RAS was subjected to regulation in pancreatic endocrine tumours, suggesting that alterations in the RAS may have pathophysiological relevance in patients with islet tumours such as pancreatic endocrine tumers (PET). In that study, the gene expressions of AT_2 receptor and angiotensinogen were significantly increased whereas the mRNA for AT_1 receptor was decreased in human PET samples [Lam and Leung 2002]. Studies with ACE inhibitor also showed that the pancreatic RAS may play a pivotal role in regulating islet blood perfusion, thereby affecting insulin release by the islet cells [Carlsson *et al.* 1998]. In this study, a specific ACE inhibitor enalaprilat and the Ang II antagonist Saralasin were administered to the isolated perfused rat pancreas. Interestingly, an increase in whole pancreatic blood flow, notably the microcirculation of islet blood flow, was noted. Addition of Ang II induced a marked vasoconstriction, which could delay the first phase of insulin release in response to glucose. Furthermore, a sustained decrease in tissue oxygen tension was detected in pancreatic islet transplants [Carlsson *et al.* 1998] while chronic hypoxia upregulated the pancreatic RAS components [Chan *et al.* 2000] and

enhanced the oxidative stress in the pancreas [Ip *et al.* 2002]. In this condition, an increased sensitivity to RAS components by chronic hypoxia should be deleterious for islet graft function. This might be due to a reduction in blood perfusion of islet transplants and/or an enhanced production of reactive oxygen species (ROS) in islet transplants. Other studies has also shown that cerulein-induced acute pancreatitis could upregulate the local pancreatic RAS [Leung *et al.* 2000]. Administration of Saralasin attenuated the oxidative stress and tissue injury following cerulein-induced pancreatitis [Ip *et al.* 2003]. These data suggest that an activation of pancreatic RAS such as in acute pancreatitis or in islet transplant appears to be closely associated with the generation of oxidative stress within the pancreas, which in turn may lead to disturbances in pancreatic functions. Emerging data also shown that patients treated with the ACE inhibitors due to a high risk of cardiovascular events, result in a marked reduction in the incidence of diabetes and development of diabetes complications [Yusuf *et al.* 2000]. Taken together, the potential influence of pancreatic RAS exert on cellular growth in exocrine acinar cells and on the generation of free radicals in islet transplantation and other clinical conditions. The significance of a local pancreatic RAS could provide an insight into several exocrine and endocrine pancreatic diseases, such as pancreatitis, pancreatic cancer, islet

transplant failure and diabetes mellitus [Leung 2003].

1.3 Islet Transplantation and RAS

1.3.1 Whole pancreas and islet transplantation

For decades, researchers have searched for ways to restore blood sugar control through transplanting pieces or extracts of pancreas in patients with diabetes. Apart from the daily need for multiple insulin injections and blood glucose monitoring, patients with type 1 diabetes mellitus (T1DM) are at greater risk for end organ complications, cardiovascular disease, and premature mortality. Treatment aimed at keeping the blood sugar, blood pressure, and blood lipids within their normal ranges has been shown to markedly reduce diabetes complications [Gaede *et al.* 2003]. However, few treatments have achieved these goals for long term. The main reasons for transplantation therapies are thus to overcome the lifelong need for daily insulin injections and frequent blood glucose testing, to promote maintenance of near normal blood glucose levels, and to avoid or even reverse the long-term diabetes associated complications.

The first known transplantation report was in 1895, in which minced sheep's

pancreas and extracts of pancreas were used for oral and subcutaneous therapy [Williams 1894]. This report turned out to be a failure in the lack of immunosuppression. No successful case was reported until 1972, when islet isografts from normal rats were used to reverse streptozotocin-induced diabetes in rats [Ballinger and Lacy 1972]. After that, successful islet autograft transplantation was reported in humans [Largiader *et al.* 1980]. In this autograft transplantation, the removed pancreas was minced, digested with collagenase, and centrifuged to separate islets from exocrine cells and infused the islets into the patient's liver through the portal venous circulation. Since the "Edmonton protocol" was published [Shapiro *et al.* 2000], researchers at the University of Alberta in Edmonton, Canada, have continued to use this new procedure to transplant pancreatic islets into patients with T1DM. The "Edmonton protocol" is a method using larger quantity of islets and a combination of drugs such as sirolimus, tacrolimus and daclizumab, which were less toxic to suppress the immune system. The largest and most recent report on the clinical outcomes of the Edmonton experience was published in 2002 [Ryan *et al.* 2002]. The authors reported 54 islet infusions in 30 recipients with type 1 diabetes and provided detailed follow-up data on 17 consecutive patients, all of whom became insulin-independent. These reports confirmed that pancreatic

islets, isolated from brain-dead donor pancreata and infused into a recipient's portal vein, can secrete insulin in a manner sufficient to regulate the blood sugar.

The goal of pancreas or islet transplantation is to achieve glycemic control with minimal risks. The significant risks associated with whole organ transplantation usually limit its use to co-transplantation with other organs. Pancreas transplantation is still associated with significant morbidity in terms of surgical risk and of cost. Islet transplantation, a much less invasive procedure than whole-pancreas transplantation, offers the hope to cure diabetes [Ryan *et al.* 2001].

1.3.2 Problems encountered in islet transplantation

Although islet transplantation can lead to insulin independency in patients with T1DM, the long-term outcome so far is disappointing. One of the major obstacles is the limited availability of human islet tissue. In the United States, there are approximately 1 million patients with T1DM and 16 million with type 2 diabetes who may wait for islet transplantation. However, only about 6000 human brain dead donors are suitable for organ donation each year [United Network for Organ Sharing 2002]. Despite all the limitations associated with whole pancreas

transplantation, there are still about 80 % of the best donors used for whole pancreas transplantation. Furthermore, even the most experienced islet isolation center can only have 50 % chance to isolate islets with good quality and quantity. Taken these into consideration, if one recognizes that almost all islet recipients currently require islets from 2-4 donors, only less than 1000 recipients per year (out of the 17 million in need) could benefit from an islet transplant. Clearly, a replenishable cellular source of physiologically regulated insulin secretion will be required.

On the other hand, rejection is another problem with islet transplantation. The immune system is programmed to destroy bacteria, viruses, and tissue it recognizes as "foreign," including transplanted islets. Immunosuppressive drugs are needed to keep the transplanted islets functioning. These drugs are often associated with serious side effects. Therefore, patients also contend with a higher risk for infections resulting from a weakened immune system. To solve this problem, some researchers are attempting to encapsulate either allogeneic or xenogeneic islets behind a barrier that allows glucose to enter the islet β cells and secrete insulin without immune detection and immune mediated destruction [Dickson *et al.* 2003; Kobayashi *et al.* 2003].

In addition to the above two major hurdles for islet transplantation, there are many

more barriers to be overcome, such as imperfect glycaemia control, unknown durability of transplanted islet function, unknown safety of intraportal islets, unknown long-term effects on secondary complications and last but not least, difficulties in identifying the ideal islet or pancreas candidate.

1.3.3 Potential role of RAS in islet transplantation

The reason for the need of large amount of islets is poor or inadequate engraftment of the islets in the implantation organ. Normally, pancreatic islets have a rich blood supply which is of crucial importance for the delivery of oxygen and nutrients to the islet cells and for the dispersal of the secreted hormones to their target organs [Jansson 1994]. However, the islet vasculture disrupts when they are isolated and cultured before transplantation [Parr *et al.* 1980]. Thus after transplantation, the islets are solely by diffusion from blood vessels in the surrounding tissues [Davalli *et al.* 1996]. Furthermore, a markedly decreased oxygen tension in islets transplanted 1 month post-transplantation was observed [Carlsson *et al.*1998] and the newly formed blood vessels were immature [Lukinius *et al.* 1995]. Clearly, attempts to improve islet revascularization are urgently needed to decrease the number of transplanted islets to obtain insulin independence. The pancreatic RAS is shown to

be responsive to a number of physiological and pathophysiological stimuli, such as chronically hypoxic condition and during other clinical conditions, e.g. acute pancreatitis [Leung and Carlsson 2001]. It was shown that infusion of Ang II induced a dose-dependent reduction in both whole pancreatic and islet blood flow, which was most pronounced in the former. Administration of enalaprilate, an inhibitor of angiotensin-converting enzyme, and saralasin, a nonselective Ang II receptor antagonist, preferentially increased islet blood flow [Calrlsson *et al.* 1998]. From these results, it could be speculated that increased local levels of RAS components in islet grafts may also occur to support islet revascularization and to decrease oxygenation between islets in the graft. The significance of local RAS in the pancreas and its regulation by inflammation and low oxygen tension conditions could be of potential importance in the study of islet transplantation. The target for the inhibition of pancreatic or islet RAS by virtue of using specific RAS inhibitors could also provide an insight into islet transplantation.

1.4 Diabetes Mellitus and RAS

1.4.1 Diabetes Mellitus

Diabetes mellitus is a group of metabolic disorders with one common manifestation: hyperglycemia caused by insulin deficiency. Diabetes mellitus is by far the most common endocrine disorder and is a worldwide health problem. Diabetes is the third leading medical cause of death and the second leading cause of blindness in the United States. This disease is not a single disease as was once thought. Instead, it is now well known that diabetes comprises a heterogeneous group of disorders that differ in both cause and severity. Several immediate complications can arise if the glucose concentration in the blood is not checked by insulin. These problems include hyperglycemia, ketoacidosis and electrolyte imbalance. When hyperglycemia becomes severe, the concentration of glucose in the blood can exceed the capacity of the kidneys to recapture glucose by active transport; glucose will be excreted in the urine, thus resulting in glucosuria. Without insulin, the unopposed actions of glucagon result in increased ketone formation by the liver. The primary ketones are organic acids, i.e. acetoacetate, β-hydorxybutyrate and acetone. When they are produced in sufficiently large amounts, the normal acid/base balance in the body is considerably disturbed, resulting in the condition termed ketoacidosis.

Ketones also carry cations, such as sodium and potassium into the urine, and thus concomitant with severe ketoacidosis is a loss of these ions. This results in an electrolyte imbalance in the body. If left untreated, severe ketoacidosis accompanied by dehydration can rapidly lead to coma and death. In addition to acute complications, long-term complications of diabetes may occur. These secondary complications often involve gradual changes that develop over a period of years and may shorten the life expectancy of the patients. The most common secondary complications are seen within the vascular system, such as narrowing of larger blood vessels in the brain, heart, and lower extremities. The resulting reduction in circulation to these areas may result in stroke, heart attack, or loss of limb. Lesions in the retina of the eye can result in eye disease termed diabetic retinopathy. Another common secondary complication involves impairment in nerve function termed diabetic neuropathy, resulting in abnormalities in bladder or gastrointestinal function.

Diabetes mellitus is classified into two types based on clinical symptoms and specific etiologic characterization.

1.4.2 Type 1 diabetes and its animal models

T1DM (also known as insulin-dependent diabetes; 5 % to 10 % of all cases) is associated with a specific and complete loss of pancreatic β cells, leaving islets composed of an increased number of α, δ and F cells. Thus, T1DM can be thought of as a specific β-cytectomy, a phenomenon mimicked in animals with the use of chemical agents like alloxan or streptozotocin (STZ). Autoimmune destruction of pancreatic β cells has been suggested to be the most common cause of T1DM. Other initiating factors include virus and chemical toxins. Less common causes of T1DM are conditions that result in a reduction in the mass of islet cell tissue, as observed in several types of pancreatitis, pancreatic carcinoma, and pancreatectomy. Exogenous insulin is required to reverse the high glucose state. Patients with T1DM must receive daily insulin injections.

Animal models featuring physiological and pathological changes characteristic of each diabetes subtype are important to understand this complex disease better and to propose potential treatments. Specific etiological factors and/or genetic backgrounds can be selected and combined to produce a particular type of experimental diabetes. This allows the researcher to explore particular biochemical

or anatomical alterations and thus to study disturbances found in human diabetes.

Chemically induced T1DM is the most commonly used animal model of diabetes. The chemical agents used specifically damage β cells, cause temporary inhibition of insulin production, and diminish the metabolic efficacy of insulin in target tissues. In general, chemicals that damage β cells are of specific interest as they closely reproduce lesions that occur during β cell destruction in T1DM. Moreover, these agents provide relatively permanent diabetes that is suitable for long-term studies. Alloxan, a cyclic urea analog was the first agent in this category to produce permanent diabetes in laboratory animals [Dunn *et al.* 1943]. STZ has replaced alloxan later as the principal agent due to the greater selectivity of β cells and the lower mortality rate seen in STZ diabetic animals [Rakieten *et al.* 1963]. Genetic models of T1DM include the NOD mouse and the BB diabetic rat. In these animals, diabetes occurs spontaneously with a total dependence on exogenous insulin for survival.

1.4.3 Type 2 diabetes and its animal models

Type 2 diabetes (T2DM, also know as non-insulin-dependent diabetes; represent 90 to 95 % of the diabetic population). T2DM is due to pancreatic β cell dysfunction and/or insulin resistance. Normal or often higher-than-normal concentrations of insulin may be present in the blood, but cells of target tissues such as adipose and muscle simply do not respond to the hormone as they normally would, resulting in a condition called insulin resistance. Nearly 90 % of the patients with T2DM are also obese. The exact relationship between the obesity and insulin resistance is not clear, but the severity of the disease can often be reduced considerably if the patients are placed on a diet and lose weight. In addition to diet, persons with T2DM are often treated with oral hypoglycemic agents known as sulfonylureas, which stimulate insulin secretion from β cells. Sulfonylureas also appear to augment the actions of insulin in tissues and therefore help overcome the insulin resistance in target tissues. Current research strongly suggests that this disease arises as a consequence of failure of insulin action due to abnormalities at the cell surface or within the cell, deficiency in insulin secretion or a combination of these processes.

The genetic T2DM models are produced though selective breeding, spontaneous mutations or genetic engineering. One example is the db/db mouse, in which diabetes mutation is a result of a point mutation in the diabetic gene, db, which encodes a leptin receptor, Lepr [Chen *et al.* 1996]. Other genetic models include ob/ob mouse, KK mouse, NZO mouse, fa/fa Zucker rat and fa/fa diabetic Zucker rat. Most of these models demonstrate various degrees of glycemia, insulinemia and obesity. Chemically induced T2DM is obtained through injection of agents that produce the desired pathology. The most commonly used is STZ. A mild and stable form of diabetes, resembling type 2 human diabetes, is produced by a single dose of STZ (90 mg/kg i.v.) in neonatal rats [Bonneve *et al.* 1981]. The induced β cell injury is followed by limited regeneration, primarily as a result of ductal budding rather than mitosis of pre-existing β cell, creating a short-term normalization of glycemia. At 6 to 15 weeks of age, the rats have an impaired glucose disposal rate and significant β cell secretory dysfunction. Surgical T2DM can also be produced by removal of various amounts of pancreatic mass.

1.4.4 RAS blockade in diabetes patients

Diabetes is a common cause of end-stage renal disease and cardiovascular disease, both of which are major public health issues. One of the mechanisms of blood pressure elevation in renal disease is overactivity of the RAS. ACE inhibitors such as ramipril and captopril prevent the formation of Ang II, a molecule that raises blood pressure by vasoconstriction of arterial vessels. A newer class of drugs to treat hypertension is the Ang II receptor blockers (ARBs). These agents, such as irbesartan and losartan, work by inducing blockade of Ang II binding to its AT_1 receptors. Several studies have demonstrated the benefits of RAS blockade in reducing blood pressure and cardiovascular and renal risk. More recent clinical trials suggested that blockade of the RAS, either by inhibiting the ACE [Hansson *et al.* 1999] or by blocking the AT_1 receptor [Dahlof *et al.* 2002], can reduce cardiovascular and renal functions independently of their antihypertensive effects. In the Captopril Primary Prevention Project (CAPPP) trial, incidence of diabetes was 14 % lower in the captopril group than in the conventional group [Hansson *et al.* 1999], whereas, in the Heart Outcomes Prevention Evaluation (HOPE) trial, there was 34 % reduction in relative risk for the development of type 2 diabetes [Yusuf *et al.* 2001]. Similarly, in the Losartan Intervention For Endpoint Reduction in

Hypertension study (LIFE), the incidence of type 2 diabetes was reduced by 25 % in the losartan group [Dahlof *et al.* 2002]. Furthermore, the RAS blockade has also been shown to reduce the risk of renal disease in diabetes. A previous study involving patients with type 1 diabetes showed that captopril, an ACE inhibitor, could reduce the risk of renal disease progression by 50 %. New trials with the Ang II receptor blockers examining the potential benefits of these newer agents in type 2 diabetic patients were initiated on the basis of the results of the captopril study. [Lewis *et al.* 1993].

1.4.5 Potential role of RAS in diabetes mellitus

Several regulatory systems control the islet function and β cell mass by means of neural, hormonal, and paracrine/autocrine/intracrine pathways. Of great interest in this context might be some paracrine/autocrine regulators such as angiotensins. Recent evidence exists for the presence of a local RAS in the pancreas, which is subject to activation by various conditions. Such a local islet RAS, if activated, could result not only in the reduction of islet blood flow, but also in the direct

inhibition of islet insulin secretion and in a subsequent deterioration of the β cell mass.

Concerning type 1 diabetes, profound effects of Ang II on blood flow and insulin release in transplanted islet has been observed [Olsson *et al.* 2000]. It may be speculated that hyperactivity of the RAS in islet vasculature impairs insulin release. Indeed, in the diabetic state, increased ACE concentrations occur in the mesenteric vasculature, at least in animals [Jandeleit *et al.* 1992]. An increased vasopressor responsiveness to Ang II in diabetic patients has also been observed [Christlieb *et al.* 1976; Drury *et al.* 1984].

Even though ACE-inhibition has shown beneficial effects on islet function in several clinical studies, whether this improved insulin secretion response reflects vascular effects in the islets, or is mediated via Ang II receptors on β cells remains to be. elucidated. The involvement of the islet RAS for the close correlation that exists between hypertension and type 2 diabetes in the clinical trials, emerges as a potential link between RAS and pancreatic islet functions. This also provides a potential mechanism for the observed beneficial effects of RAS inhibition in reducing the diabetes incidence in at-risk patients with hypertension in large clinical trials. In

the future, it will therefore be important to investigate more closely the role of the islet RAS in human diabetes, especially with regard to effects on β cells and insulin secretion.

1.5 Aims of study

Based on the available information described in the introduction, the present study is, specifically, designed to investigate the existence of a islet RAS in the pancreas: its role in insulin secretion, in islet transplantation and in diabetes. Specifically, the aims of present study are set as follows:

- Existence of a local RAS in pancreatic islets
- Role of islet RAS in insulin secretion
- Regulation of islet RAS by islet transplantation
- Role of islet RAS in insulin secretion in T2DM
- Regulation of islet RAS by T2DM

Chapter 2 Materials and Methods

2.1 Experimental animals and mouse models

2.1.1 Experimental animals for islet isolation and transplantation

Male C57BL/6J mice aged 8-10 weeks were used in all experiments. The animals were bred in the Laboratory Animal Services Center of the Chinese University of Hong Kong, fed with standard rat chow, and supplied with water *ad libitum*. The mice were housed under a 12-hour light/dark cycle with constant ambient temperature of 22 °C ± 1 °C and relative humidity in the range of 60 % to 80 %. The experimental procedures were approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong and followed the "Principles of laboratory animal care" (NIH publication no. 85-23, revised 1985). The animals were subjected to pentobarbital-anesthetic management when needed during the whole period of experiment and were killed by cardiac excision.

2.1.2 Mouse model for type 2 diabetes

6-8 weeks genetically diabetic C57BL/KsJ+db/+db mice and their age matched nondiabetic littermates C57BL/Ksjm+/+db are used in experiments for the regulation of pancreatic islet RAS in type 2 diabetes.

The C57BL/KsJ+db/+db mice (common name db/db mice) has a point mutation in the diabetic gene, db, which encodes a leptin receptor, Lepr [Chen *et al.* 1996]. Leptin is a recently isolated hormone produced by adipocytes and is a powerful regulator of satiety centers in the brain. A defect in either leptin production or transmission of the leptin signal in db/db mice, respectively, results in a syndrome of obesity and diabetes which closely resembles that which occurs in humans. Leptin release is regulated in part by nutritional status and its expression in adipose tissue is up-regulated by insulin. The control animals are nondiabetic heterozygous C57BL/Ksj-m+/+db mice (common name m+/+db mice).

The animals were bred in the Laboratory Animal Services Centre of the Chinese University of Hong Kong and housed as the same condition as that mentioned in the last section.

2.2 Islet isolation and transplantation

2.2.1 Enzymatic islet isolation

Islets were isolated from the pancreas as described previously [Andersson 1978]. Briefly, male C57BL/6J mice aged 10 weeks were killed by cervical dislocation and the pancreata were dissected out. The pancreata were then placed in cold Hanks' solution (Sigma Aldrich, St. Louis, USA) and injected with Hanks' solution by means of a 27 G syringe. The inflated pancreata were cut into small pieces with similar size. These pieces were transferred to vials containing collagenase solution (25 mg/8ml Hanks) (Roche Molecular Biochemicals, Mannheim, Germany). The vials were shaken vigorously at 37 °C for approximately 15 minutes. The collagenase digestion was terminated by the addition of cold Hanks' solution and the digest was then washed three times by filling the vial with Hanks. The islets were then picked using mouth-pipettes and cultured free-floating for 4-7 days in non-adherent culture dishes using RPMI 1640 medium (Sigma Aldrich, St Louis, USA) supplemented with 10 % (vol/vol) fetal bovine serum (Sigma Aldrich). Medium was changed every other day.

2.2.2 Islet transplantation

The method for islet transplantation has been described previously [Carlsson *et al.* 1998]. Briefly, 300 isolated islets were packed into the tip of the mouth-pipette after 4-7 days of culture. A recipient C57BL/6J mouse was anesthetized with an i.p. injection of 2.5 % (wt/vol) pentobarbital and a small incision was made on the left renal capsule. The tip of the mouth-pipette was introduced between the renal parenchyma and the capsule followed by gentle ejection of the pellet. Four weeks after transplantation, the mice were killed and the islet transplant was dissected out. As control, pancreatic islets isolated from age-matched animals were used.

2.3 Biological assay on islet functions

2.3.1 Measurement of islet insulin release

Measurement of islet insulin release was carried out following the procedure as described previously [Wang and Gleichmann 1998]. Groups of ten islets were transferred in duplicate to Falcon 24-well culture plates containing 0.25ml Krebs-Ringer bicarbonate buffer (KRBB) supplemented with 10mmol/l HEPES and 2 mg/ml of bovine serum albumin (BSA). For the first hour of incubation at 37 °C

(O_2/CO_2, 95:5), the medium contained 1.7 mmol/l glucose. The medium was then removed and replaced with 0.25 ml of KRBB containing 16.7 mmol/l glucose and incubated for another hour. The effect of Ang II (Sigma Aldrich,) on insulin release from isolated islets was determined at 0.1, 1, 10 and 100 nmol/l of Ang II. Ang II was added in the KRBB medium with 16.7 mmol/l glucose during the second hour of incubation. Specific Ang II-receptor antagonists for AT_1, losartan (Merck & Co., NJ, USA; 1 μM) and for AT_2, PD123319 (Sigma Co., St. Louis, MO, USA; 1 μM) were administered 10 min before the Ang II treatment. After the incubation, the medium was collected for the measurement of insulin release using a Mouse Insulin ELISA Kit (Mercodia, Uppsala, Sweden). Figure 2-1 is a schematic diagram of the procedure for measurement of insulin release.

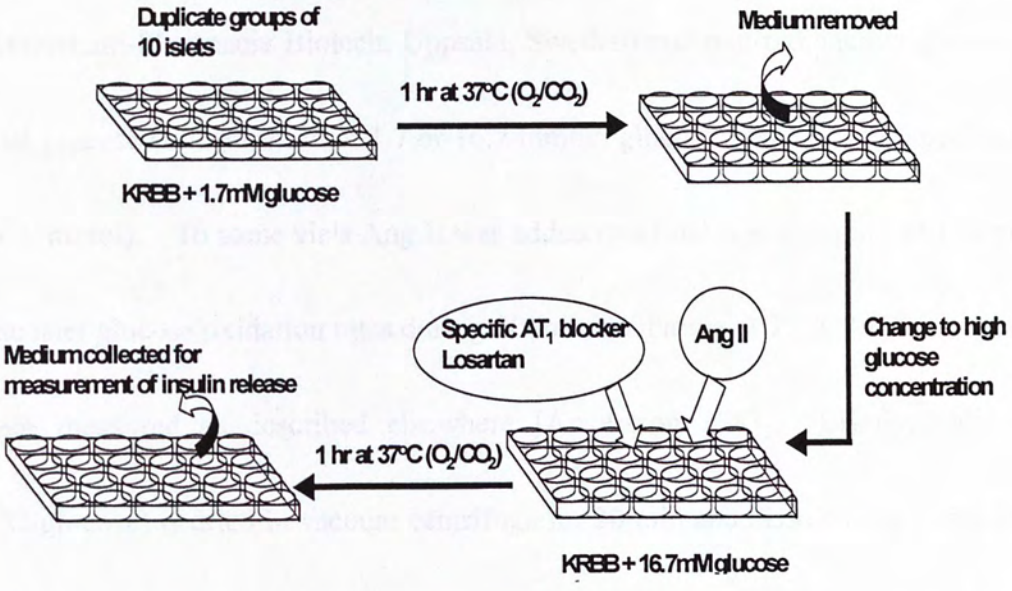


Figure 2-1 Schematic diagram of measurement of insulin release

2.3.2 Measurement of islet glucose oxidation rate

Triplicate groups of ten islets were transferred to glass vials containing 100 µl of Kerbs Ringer Bicarbonate Buffer (KRBB) supplemented with D-[U-^{14}C]glucose (Amersham-Pharmacia Biotech, Uppsala, Sweden) and non-radioactive glucose to a final glucose concentration of 1.7 or 16.7 mmol/l glucose, respectively (spec. act. 0.5 mCi/ mmol). To some vials Ang II was added to a final concentration of 100 nmol/l. The islet glucose oxidation rates during 90 min incubation at 37 °C (95 %O_2/5 %CO_2) were measured as described elsewhere [Andersson 1983]. Briefly, radioactive (^{14}C-glucose) is dried in vacuum centrifuge for 30 min and dissolved in 1.2ml low or high glucose solution. 100 μl of the radioactive glucose solution was put into each Jarret vial. AngII (100nmol/l) and/or losartan (1 μmol/l) was added in both low and high glucose concentration vials. Islets were picked and washed in Hanks' solution and then 10 islets were put into each Jarret vial (triplicate each set). The Jarret vials were put into dry, clean glass vials that were gassed with (95 % O_2/5 % CO_2), covered with lids. The vials were then incubated in 37 °C water bath with shaking for 90 min. After incubation, 100 μl of antimycin (Sigma Co., St. Louis, MO, USA) was injected into the Jarret vials with islets slowly, using a 27G needle and 1ml syringe to kill the β cells. Then 250 μl of hyamine (ICN Biomedicals Inc.,

Aurora, OH, USA) was injected into the large glass vials with a 18G needle and 1ml syringe to trap the CO_2. Lastly, 100 μl phosphate buffer(pH 6.0): was injected into the Jarret vials with islets using a 27G needle and 1ml syringe to lower the pH. The vials were then incubated in 37 °C water bath for another 120 min (without shaking). 5 μl of the radioactive glucose solution (both low and high concentration) and 250 μl hyamine were put into 3 vials for standard control. After incubation, the Jarret vials were removed, and the glass vials filled with 5ml Ultima Gold (PerkinElmer Life Sciences, Boston, MA, USA) scintillation liquid. Amount of radioactive glucose oxidized was measured using a scintillation counter. Calculation of oxidized glucose content was shown as follow:

1. Transformation factor = 5 x 16700 / *dpm Standard (pmol/dpm)
2. Amount of oxidized glucose (pmol)
   = (dpm Sample – dpm Blank) x transformation factor

*dpm=degradation per minute

Figure 2-2 is a schematic diagram of the measurement of glucose oxidation

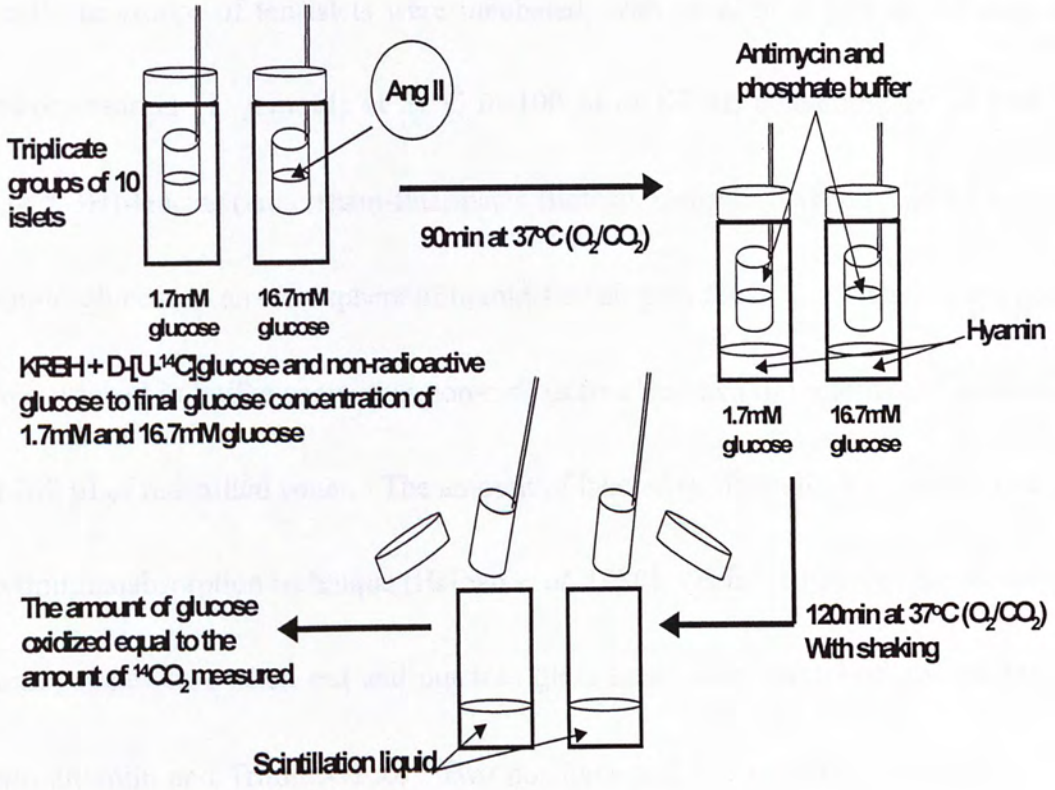


Figure 2-2. Schematic diagram for the measurement of glucose oxidation.

2.3.3 Measurement of islet (pro)insulin biosynthesis

Duplicate groups of ten islets were incubated, with or without 100 nmol/l Ang II, and/or losartan (1 μmol/l) at 37°C in 100 µl of KRBB containing 50 µCi/ml of L-[4,5-3H]-leucine (Amersham-Pharmacia Biotech, Uppsala, Sweden) and 1.7 or 16.7 mmol/l glucose in an atmosphere of humidified air plus 5%CO_2. After 2 h, the islets were washed in buffer containing non-radioactive leucine (10 mmol/l) and sonicated in 200 µl of redistilled water. The amount of labeled (pro)insulin was determined by an immunoabsorption technique [Halban *et al.* 1980]. Briefly, 10 μl of the sonicated homogenates was taken out and put into glass tubes with 100 μl of glycine buffer with albumin and Triton X-100. Two positives and two negatives were set up for each sample. For the positives, 10 μl of anti-insulin serum (Chemicon Internation Inc., Temecula, CA, USA) was added and for the negatives, 10 μl of normal guinea pig serum was added. The samples were incubated for 1 hour in 37°C. Then, 50 μl of rProtein A Sepharose (Amersham Biosciences, Uppsala, Sweden) was added to the samples and shaked in room temperature for 15 minutes. The samples were then centrifuged and washed twice with 500 μl Glycine buffer. The pellets were resuspend in 500 μl Acetic acid with albumin and then poured into 6ml scintillation flasks. 4ml Ultima Gold (PerkinElmer Life Sciences, Boston, MA, USA)

scintillation liquid was added for each flask and the radioactivity counted by a scintillation counter.

2.3.4 Measurement of Islet total protein synthesis

As for insulin biosynthesis, duplicate groups of ten islets were incubated, with or without 100 nmol/l Ang II, and/or losartan (1 μ mol/l) at 37 °C in 100 µl of KRBB containing 50 µCi/ml of L-[4,5-3H]-leucine (Amersham-Pharmacia Biotech, Uppsala, Sweden) and 1.7 or 16.7 mmol/l glucose in an atmosphere of humidified air plus 5 %CO_2. After 2 h, the islets were washed in buffer containing non-radioactive leucine (10 mmol/l) and sonicated in 200 µl of redistilled water. The total protein biosynthesis was measured in trichloroacetic acid precipitates of the islet homogenate [Halban *et al.* 1980]. The samples after sonication were centrifuged and washed once with 500 μ l glycine buffer. The pellets were resuspend in 500 μ l acetic acid with albumin and then poured into 6ml scintillation flasks. 4ml Ultima Gold (PerkinElmer Life Sciences, Boston, MA, USA) scintillation liquid was added for each flask and the radioactivity counted by a scintillation counter. Figure 2-3 shows a schematic diagram for the measurement of islet (pro)insulin and protein biosynthesis.

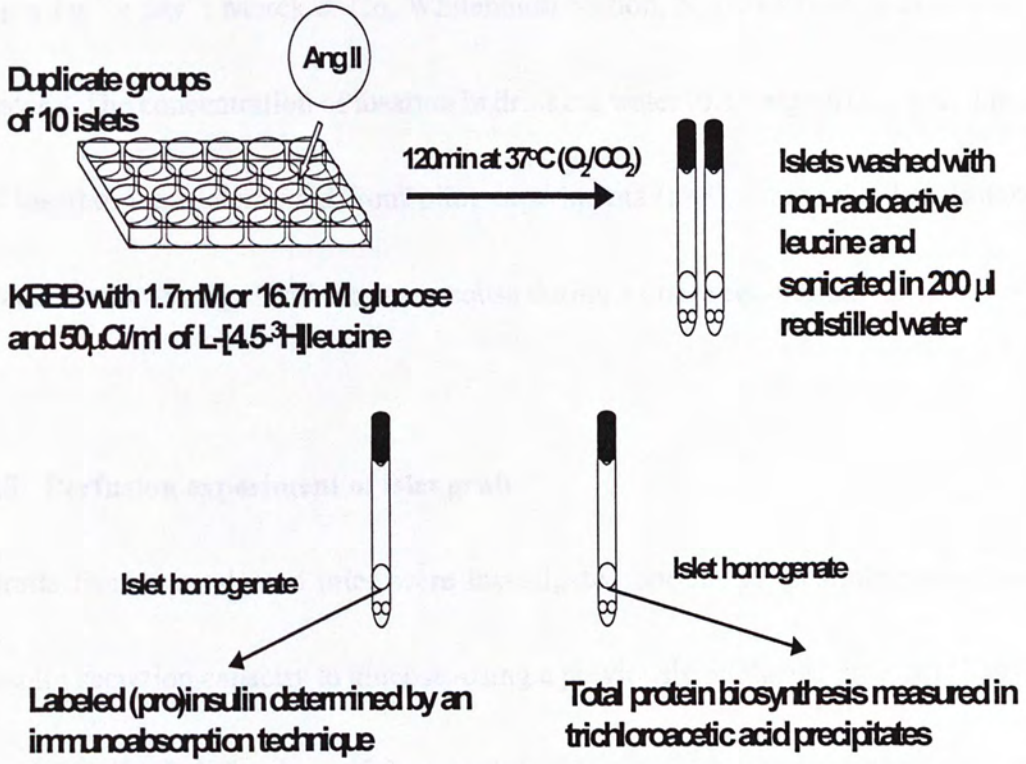


Figure 2-3 Schematic diagram for measurement of islet (pro)insulin and protein biosynthesis

2.4 Chronic losartan treatment

Some of the transplanted mice were in random assigned to treatment with losartan (30 mg x kg^{-1} x day^{-1}; Merck & Co, Whitehouse Station, NJ) dissolved In their drinking water. The concentration of losartan in drinking water (0.13 mg/ml) to yield this dose of losartan was determined from pilot experiments (n=3), where the daily intake of water was in average 7 ml for each mouse during a one-week-period.

2.5 Perfusion experiment of islet graft

Grafts from transplanted mice were investigated one month posttransplantation for insulin secretion capacity to glucose, using a previously published protocol [Korsgren *et al.* 1989]. Briefly, the graft-bearing left kidney was removed together with part of the aorta and inferior vena cava. The ureter and the renal vein were cut, whereas the aorta was cannulated and infused with a continuously gassed (O_2/CO_2 = 95:5) Krebs Ringer Bicarbonate Buffer (KRBB) supplemented with 2% (wt/vol) each of bovine serum albumin (BSA, fraction V, Miles Laboratories, Slough, U.K.) and dextran T70 (Pharmacia, Uppsala, Sweden) and 2.8 or 16.7 mmol/l glucose. The medium was administered at a rate of 1.0 ml/min without recycling for 60 min with a perfusion pressure of ~40 mmHg. The perfusion experiments started with a 15-min period

using a medium containing 2.8 mmol/l glucose, followed by 30-min with 16.7 mmol/l glucose and finally 15-min with 2.8 mmil/l glucose again. In some experiments, Ang II (Sigma) or losartan (Merck) were added during the perfusion with the high glucose medium (16.7 mmol/l) at a concentration of 10 ng/ml and 100 μ g/ml respectively. A 1.0 ml sample of the effluent medium was collected every fifth min, except for the first 10 min of perfusion with the high glucose concentration when samples were taken after 1-5, 7 and 10 min. The insulin concentration of the effluent samples were measured by ELISA (Mercodia, Uppsala, Sweden). The rate of insulin secretion was calculated by multiplying the insulin concentration in the sample by flow rate, giving values of insulin expressed as nanograms of insulin per minute.

2.6 Insulin content of the islet graft

After perfusion the graft was removed from the kidney and homogenized in 1000 μ l of acid-ethanol (0.18 mmol/l HCL in 95 % [vol/vol] ethanol). The samples were extracted overnight at 4 °C, followed by ELISA of insulin (Mercodia).

2.7 Islet graft (pro)insulin and total protein biosynthesis

Some of the transplanted mice were one month posttransplantation allocated to measurements of islet graft (pro)insulin and total protein biosynethesis as previously described (Section 2-3-3 & 2-3-4) [Korsgren *et al.* 1989]. Briefly, explanted islet grafts were incubated with or without 1 μ mol/l losartan (Merck), at 37 °C in 100 μl of KRBB containing 50 μ Ci/ml of L-[4,5-3H]leucine (Amersham-Pharmacia Biotech, Uppsala, Sweden) and 16.7 mmol/l glucose in an atmosphere of humidified air plus 5 % CO_2. After 2 hours, the islets were washed in buffer containing non-radioactive leucine (10 mmol/l) and sonicated in 200 μl of redistilled water. The amount of labeled (pro)insulin and total protein biosynthesis were determined as described in Section 2-3-3 and 2-3.4.

2.8 Real-time RT-PCR analysis

Real-time RT-PCR is a new technique to quantitate mRNA expression levels which requires minimal sample and no postreaction manipulation [Winer *et al.* 1999]. Briefly, real-time PCR monitors the degradation of a dual-labeled fluorescent probe (Tag-Man probe) in real time concomitant with PCR amplification. The 5' nucleolytic activity of *Taq* polymerase cleaves the probe, spatially separating the 5'

reporter fluorescent dye from the 3' quencher fluorescent dye, resulting in an increase in the emission spectra of the reporter fluorescent dye. These data are graphed as amplification plots. Figure 2-4 shows a schematic diagram of real-time PCR technique. In this study, real-time quantitative RT-PCR was performed using an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, Calif., USA) as validated before. Total RNA was extracted from pancreas or pools of all islets isolated from control and retrieved from transplanted mice (groups of 8-10 mice each), using the TRIzol reagent kit (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. Total RNA was also extracted from liver and kidney in order to study the relative abundance of islet mRNA compared to these tissues. The RNA was used as template in one-step Taq Man amplification reactions.

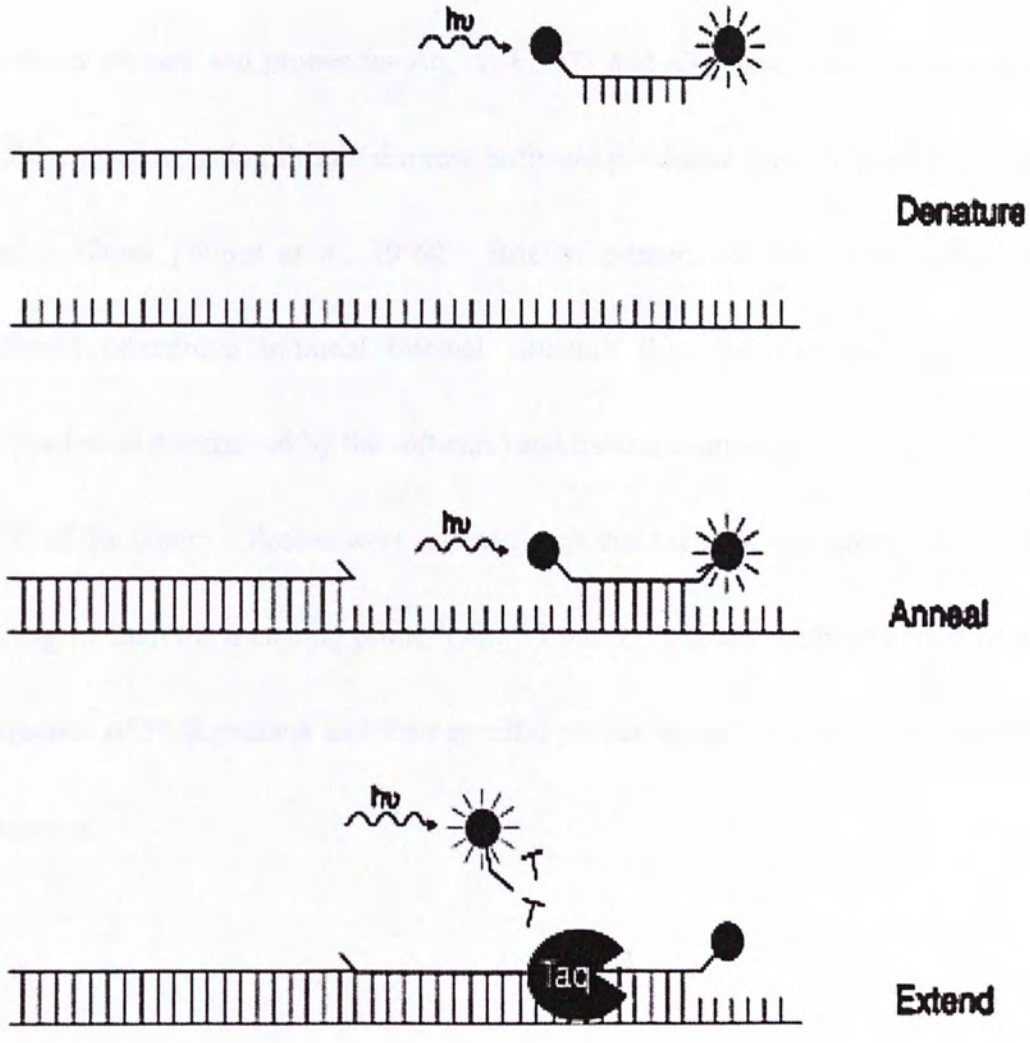


Figure 2-4. Schematic diagram showing the reaction in a real-time PCR. Dark circle: quencher fluorescent dye; gray circle: reporter fluorescent dye.

2.8.1 Design of primers and probes

TaqMan primers and probes for Ao, ACE, AT_1 and AT_2 were designed from mouse cDNA sequence using Primer Express Software purchased from Applied Biosystems Perkin-Elmer [Winer *et al.* 1999]. Briefly, primers for each gene target were selected containing minimal internal structure (i.e., hairpins and primer-dimer formation as determined by the software) and having compatible T_m (i.e., each within 1 °C of the other). Probes were selected such that their T_m was approximately 5-10 °C higher than the matching primer pair. Table 2-1 depicts the brief details of RAS sequence of PCR primers and their specific probes, as well as corresponding cDNA sequence.

Table 2-1. Sequence of specific PCR primers and fluorescent probes for the RAS components, and β-actin employed for the real-time quantitative RT-PCR. Ao denotes angiotensinogen

RAS gene	Sequence	Corresponding sequence of GeneBank
AT_1	Forward : CCA TTG TCC ACC CGA TGA AG	458-477 (s37484)
	Reverse : TGC AGG TGA CTT TGG CCAC	520-502 (s37484)
	Probe : CTC GCC TCC GCC GCA CGA	479-497 (s37484)
AT_2	Forward : CAG CAG CCG TCC TTT TGA TAA	176-196(U04828)
	Reverse : TTA TCT GAT GGT TTG TGT GAG CAA	256-233 (U04828)
	Probe : CAA CTG GCA CCA ATG AGT CCG CCT	205-228 (U04828)
ACE	Forward : TGA GAA AAG CAC GGA GGT ATC C	336-357 (M55333)
	Reverse : AGA GTT TTG AAA GTT GCT CAC ATC A	420-396 (M55333)
	Probe : ACC CTG AAA TAT GGC ACC CGG GC	364-386 (M55333)
Ao	Forward : GAG GCA AAT CTG AGC AAC ATT G	1196-1216 (NM_007428)
	Reverse : GAG TTC GAG GAG GAT GCT ATT GA	1269-1247 (NM_007428)
	Probe : TGA CAC CAA CCC CCG AGT GGG A	1218-1239 (NM_007428)
β-actin	Forward : CCG TGA AAA GAT GAC CCA GAT C	267-288 (M12481)
	Reverse : CAC AGC CTG GAT GGC TAC GT	339-320 (M12481)
	Probe : TGA GAC CTT CAA CAC CCC AGC CAT G	294-318 (M12481)

2.8.2 Use of internal control

Mouse β-actin RNA was used as internal control. The purpose of the internal control gene is to normalize the PCRs for the amount of RNA added to the reverse transcription reactions. Standard housekeeping genes such as β-actin are usually used as internal controls. The internal control gene is properly validated for each experiment to determine that gene expression is unaffected by the experimental treatment.

2.8.3 RT-PCR reaction

TaqMan reactions were set up in a reaction volume of 25 μl by using components supplied in a Taq Man PCR reagent kit. Each reaction consisted of 12.5 μl PCR master mix, 18 μmole of each amplification primer, 25 μM corresponding Taq Man probe and 30 ng RNA template. Each sample was run in duplicate with initial 30 min at 48 °C and 10 min at 95 °C for reverse transcription, followed by 40 cycles at 95 °C for 15 sec and at 60 °C for 1 min. Amplification data were collected by the 7700 Sequence Detector and analyzed by use of the Sequence Detection System software. The RNA concentration was determined from the threshold cycle (C_T) at

which fluorescence is first detected, the cycle number being inversely related to RNA concentration. The brief details of RAS sequence of PCR primers and their specific probes, as well as corresponding cDNA sequence, were listed in Table2-1.

2.8.4 Calculation using the comparative C_T method

The fold changes in RAS mRNAs by islet transplantation were calculated using the $2^{-\Delta\Delta CT}$ method [Livak and Schmittgen 2001]. In the real-time RT-PCR, a value, C_T is calculated based on the time (measured in PCR cycle numbers) at which the reporter fluorescent emission increases beyond a threshold level. The C_T value is correlated to input target mRNA levels; a greater quantity of input mRNA target results in a lower C_T value, as a result of requiring less PCR cycles for reporter fluorescent emission intensity to reach the threshold. The ΔC_T value is calculated by the subtraction of the β-actin C_T from each sample C_T. The $\Delta\Delta C_T$ value is calculated by subtraction of the control ΔC_T from each transplanted sample ΔC_T. The expression relative to control is calculated using the equation $2^{-\Delta\Delta C_T}$.

2.9 Western blot analysis

Islets were isolated from control or retrieved from transplanted mice. Protein of 300 islets was extracted using CytoBuster™ Protein Extraction Reagent (Novagen, Darmstadt, Germany). Protein content of lysates was determined by a Bio-Rad protein assay kit (Bio-Rad, Munich, Germany). Western blot analysis of AT_1-receptor protein expression was performed according to our previously described protocol [Leung *et al.* 2000]]. Briefly, proteins (10 μg/lane) were subjected to electrophoresis on a 12 % (wt/vol) polyacrylamide. The blotted protein was saturated with 5 % (wt/vol) of skimmed milk in phospate-buffered saline (PBS; pH 7.4) and 0.1 % (vol/vol) of Tween 20 for 1 hour at room temperature. The membrane was sequentially incubated in rabbit anti-AT_1 serum (1:300 dilution) overnight at 4 °C and a peroxidase-labelled anti-rabbit IgG antibody (1:1300 dilution) for 1 hour at room temperature. The positive signal was revealed using ECL plus Western blotting detection reagents and autoradiography film (Amersham, Buckinghamshire, UK). The chemiluminescence intensity of the bands was quantified using an image analyzer (Molecular Dynamics Image Quant, Sunnyvale, CA, USA).

2.10 Immunocytochemistry

Immunohistochemistry coupled with a double-staining technique [Lam and Leung 2002; Fung *et al.* 2002] was employed to determine the specific localization of AT_1-receptor in islets. Whole pancreas or isolated islets were fixed with 4 % (vol/vol) cold paraformaldehyde and embedded in paraffin. Sections (5 μm thick) were mounted on glass slides, deparaffinized and processed for indirect immunofluorescent double staining. After several washes with PBS (pH 7.4), each slide was incubated with 4 % (wt/vol) Normal Donkey serum (Jackson ImmunoResearch, PA, USA) for 60 minutes at 37 °C. Excess blocking solution was removed and the slide was incubated overnight at 4 °C with rabbit anti-AT_1 serum (Santa Cruz Biotech., Santa Cruz, CA, USA), diluted to 1:25, and with goat anti-insulin serum (Santa Cruz Biotech.), diluted to 1:50. After 3 washes with PBS, the primary antibodies were detected using an anti-rabbit antibody labeled with Rhodamine (AT_1-receptor) and an anti-goat antibody labeled with Aminomethylcoumarin Acetate (insulin) (Jackson ImmunoResearch, PA, USA) at room temperature for one hour. Preadsorption and omission of primary antibodies were used as negative controls. Positive immunostaining for AT_1 (red) and for insulin (blue) was examined with a fluorescent microscope equipped with a DC 200

digital camera (Leica Microsystems).

Semiquantitative assessment of islet β-cell mass was performed by determining the percentage proportion of area per islet section occupied by the positive staining (blue) within each islet (x40 objective). A total of 10 islets per mouse pancreas were analyzed.

2.11 Statistical data analysis

Results were expressed as means ± SEM for all groups. Multiple comparisons between data were performed using ANOVA followed by Tukey's test, or, when comparisons only were made toward control, by Dunnett's test. When only two groups were compared, probabilities (P) of chance differences between the experimental groups were calculated with Student's unpaired two-tailed t-test. For all comparisons, $P<0.05$ was considered statistically significant. For Western blot analysis, data were normalized as a percentage of control. For real-time RT-PCR, the relative expression was normalized as percentage of β-actin and calculated using the comparative C_T method of $2^{-\Delta\Delta C_T}$, as described previously.

Chapter 3 Results

3.1 Effects of Angiotensin II and Losartan on islet insulin release

3.1.1 Insulin release from normal islets

Insulin release from the isolated islets was markedly enhanced as expected when changing glucose concentration in the incubation medium from 1.7 to 16.7 mmol/l (Fig. 3-1). However, the glucose-stimulated insulin release was dose-dependently decreased by the addition of 0.1, 1, 10 and 100 nmol/l of Ang II (Fig. 3-1). At the highest used concentration of Ang II (100 nmol/l), the glucose-induced insulin release could be completely prevented. Pretreatment of isolated islets with 1 μmol/l of losartan, a specific antagonist for the AT_1-receptor, before the addition of Ang II (100 nmol/l) completely restored the insulin secretion to the glucose-stimulated level (Fig. 3-2). However, pretreatment with 1 μmol/l of PD123319, a specific antagonist for the AT_2-receptor had no effect on glucose-stimulated insulin release (data not shown). On the other hand, losartan *per se* had no effect on glucose-stimulated insulin secretion.

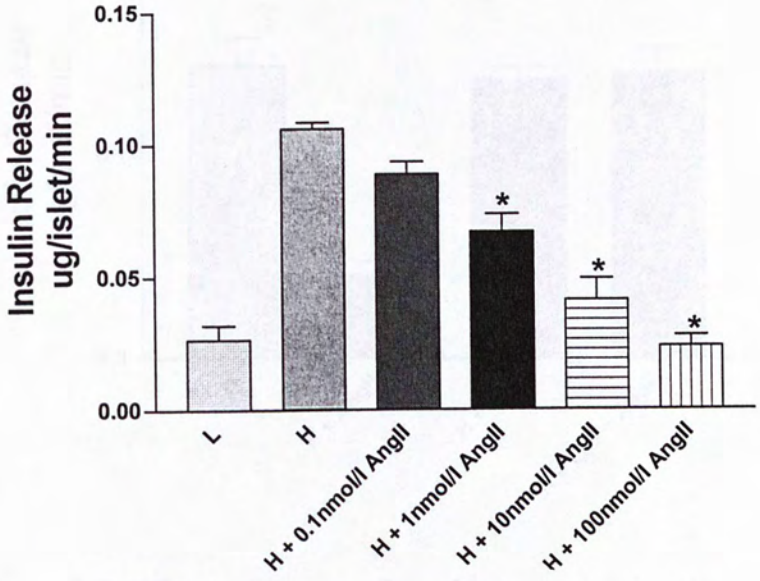


Figure 3-1. Insulin release from isolated mouse islets in the presence of 1.7 (low; L) or 16.7 mmol/l (high; H) glucose. Ang II was applied at concentrations of 0.1, 1, 10 and 100 nmol/l at the higher glucose concentration. All data are expressed as means $\pm$ SEM for four experiments in each group. * denotes $P<0.05$ when compared to islets exposed to 16.7 mmol/l glucose only.

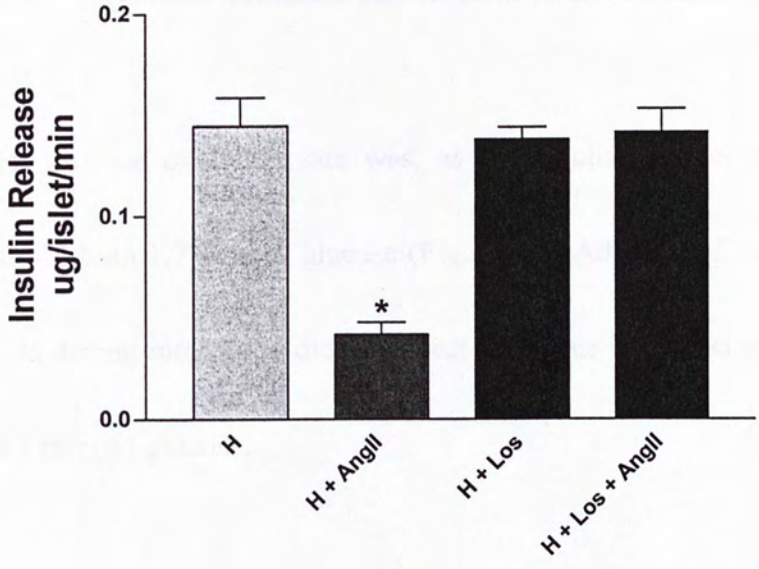


Figure 3-2. Effects of losartan (Los, 1μmol/l) and Ang II (100 nmol/l) on the glucose -stimulated (16.7 mmol/l) (high; H) insulin release from isolated islets. All data are expressed as means ± SEM for four experiments in each group. * denotes $P<0.05$ when compared to islets exposed to 16.7 mmol/l glucose only.

3.2 Effects of Angiotensin II and Losartan on islet glucose oxidation rate, (pro)insulin and total protein biysynthesis

3.2.1 Glucose oxidation rate of isolated normal islets

Islet glucose oxidation rate was, as the insulin release, markedly higher at 16.7 mmol/l than 1.7 mmol/l glucose (Fig. 3-3). Addition of Ang II (100 nmol/l) to the islets during incubation did not affect either the islet glucose oxidation rate at 1.7 or 16.7 mmol/l glucose.

3.2.2 (Pro)insulin and total protein biosynthesis of isolated normal islets

On the other hand, islet (pro)insulin and total protein biosynthesis were, as the insulin release, markedly higher at 16.7 mmol/l than 1.7 mmol/l glucose (Fig. 3-4 and Fig. 3-5). Islet (pro)insulin biosynthesis at 16.7 mmol/l glucose but not at 1.7 mmol/l glucose was inhibited by 100 nmol/l Ang II (Fig. 3-4). Islet total protein synthesis was, however, not affected by Ang II at either 1.7 or 16.7 mmol/l glucose (Fig. 3-5).

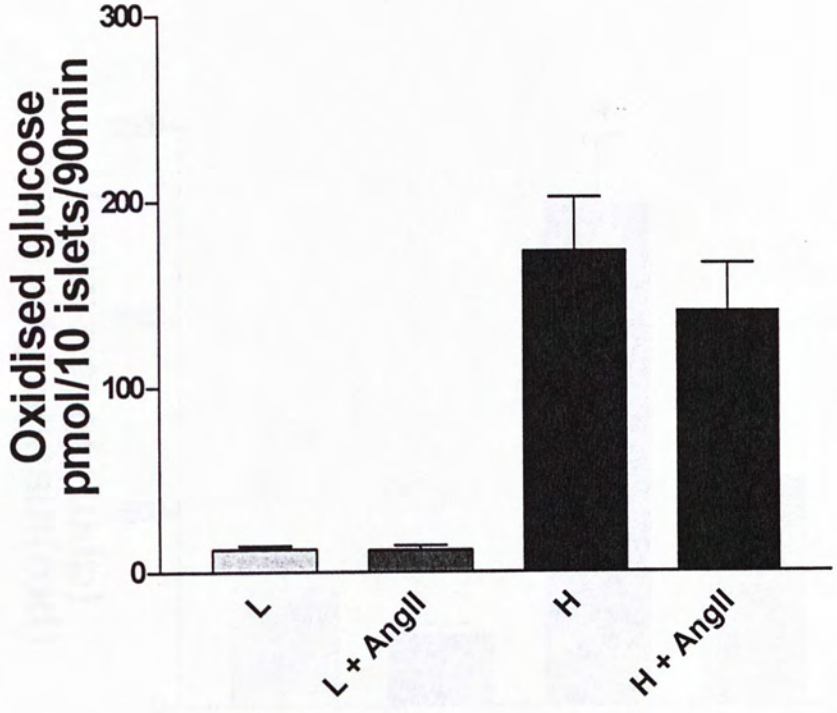


Figure 3-3. Influence of Ang II (100 nmol/l) on the glucose oxidation rate in isolated mouse islets exposed to 1.7 (low; L) or 16.7 mmol/l (high; H) glucose. All data are expressed as means±SEM for 9 experiments in each group.

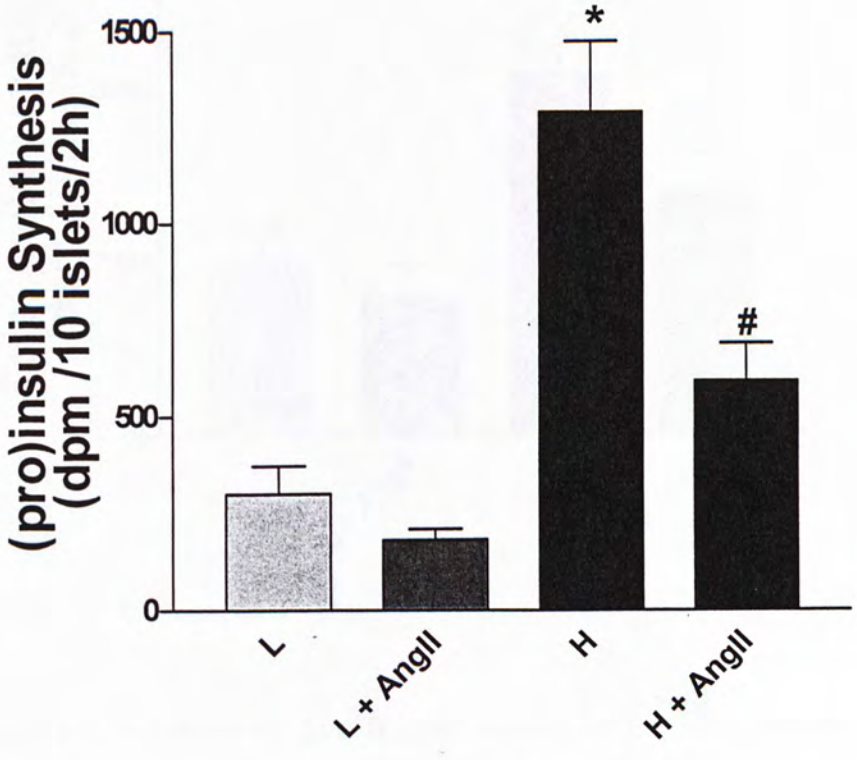


Figure 3-4. Influence of Ang II (100 nmol/l) on the (pro)insulin biosynthesis in isolated mouse islets exposed to 1.7 (low; L) or 16.7 (high; H) mmol/l glucose. All data are expressed as means±SEM for 6 experiments in each group. * denotes $P<0.05$ when compared to corresponding islets exposed to 1.7 mmol/l glucose, whereas # denotes $P<0.05$ when compared to islets exposed to 16.7 mmol/l glucose only.

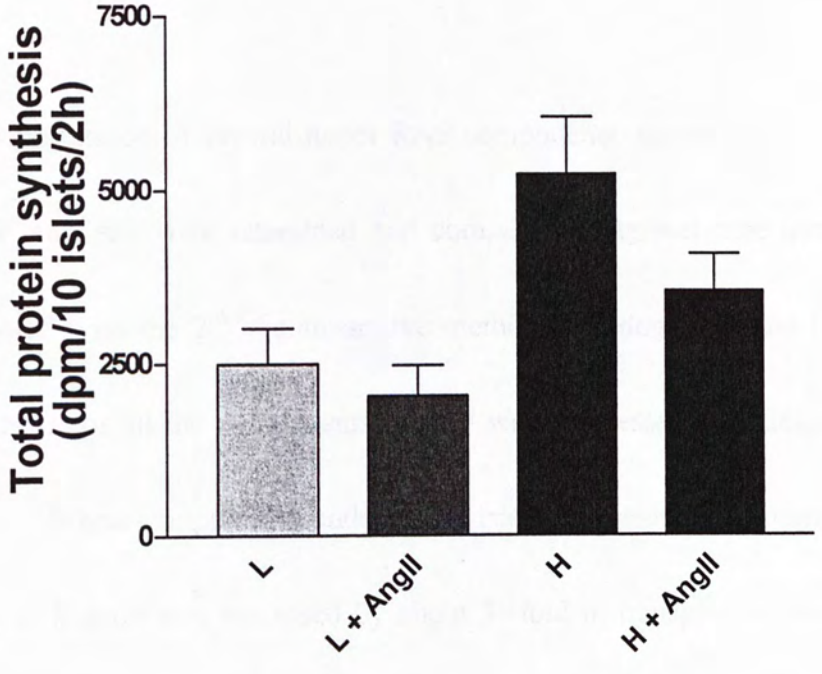


Figure 3-5. Influence of Ang II (100 nmol/l) on the total protein biosynthesis in isolated mouse islets exposed to 1.7 (low; L) or 16.7 (high; H) mmol/l glucose. All data are expressed as means±SEM for 6 experiments in each group.

3.3 Regulation of RAS components by islet transplantation

3.3.1 Expression of RAS components in endogenous islets and transplanted islets

The expression of several major RAS components, namely AT_1- and AT_2-receptors, ACE and Ao, were examined and compared using real-time quantitative RT-PCR coupled with the $2^{-\Delta\Delta C_T}$ comparative method in endogenous and transplanted islets. mRNAs for all the components studied were expressed in endogenous islets (Table 3-1). When compared to endogenous islets, the relative expression of mRNAs for AT_1 to β-actin was increased by about 3- fold in transplanted islets (Fig. 3-6). In contrast, the relative expression of mRNAs for AT_2 to β-actin was down-regulated to approximately one third in transplanted islets (Fig. 3-7). The expression of mRNA for ACE in endogenous islets did not differ significantly from transplanted islets (Fig. 3-8) while the expression of angiotensinogen mRNA in transplanted islets was lower than in endogenous islets (Fig. 3-9).

Table 3-1. Data on the expression of RAS components in endogenous and transplanted pancreatic islets using the comparative C_T method.

	RAS gene	C_T [a]	ΔC_T [b]	$\Delta\Delta C_T$ [c]	Expression relative to control [d]
Transplanted	*β-actin*	16.72 ±0.31			
	AT1	23.82±0.66	7.1±0.75	-1.47±0.72	2.77
	AT2	29.96±0.60	13.24±0.64	+1.70±1.20	0.31
	ACE	22.34±0.47	5.62±0.59	+0.94±0.88	0.52
	Ao	26.05±0.50	9.33±0.56	+1.36±0.66	0.39
Control	*β-actin*	15.00±0.62			
	AT_1	23.58±0.82	8.57±0.29		
	AT_2	26.54±0.30	11.54±0.80		
	ACE	19.69±0.60	4.69±0.75		
	Ao	22.98±0.50	7.97±0.15		

Ao Angiotensinogen

[a] The average of the C_T data for each sample

[b] The ΔC_T value is calculated by the subtraction of the β-actin C_T from each sample C_T

[c] The$\Delta\Delta C_T$ value is calculated by subtraction of the control ΔC_T from each transplanted sample ΔC_T

[d] The expression relative to control is calculated using the equation $2^{-\Delta\Delta C_T}$

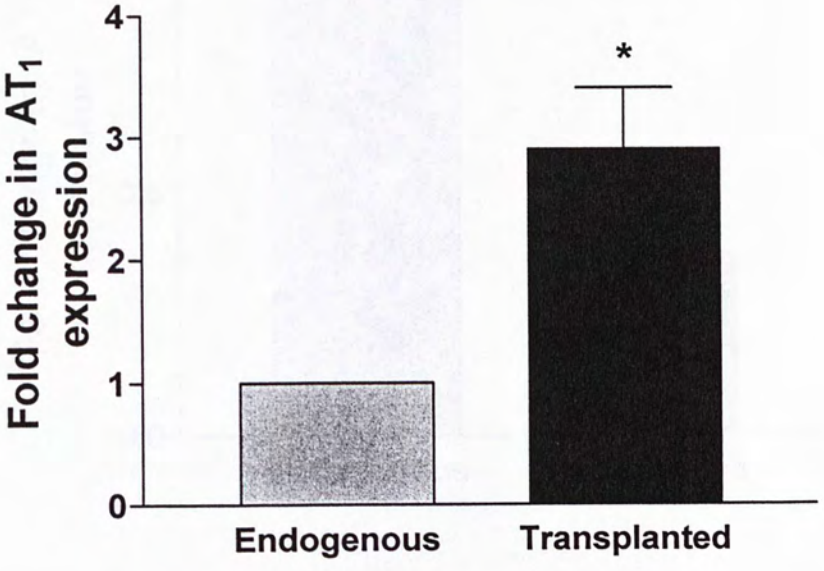


Figure 3-6. Real-time RT-PCR analysis of the mRNA expression of A. AT_1 receptor; in endogenous and transplanted mouse islets. The relative expression was normalized as percentage of β-actin calculated using the comparative C_T method of $2^{-\Delta\Delta C_T}$ (Table 2). All data are expressed as means ± SEM for four experiments in each group. * denotes $P<0.05$ when compared to endogenous islets.

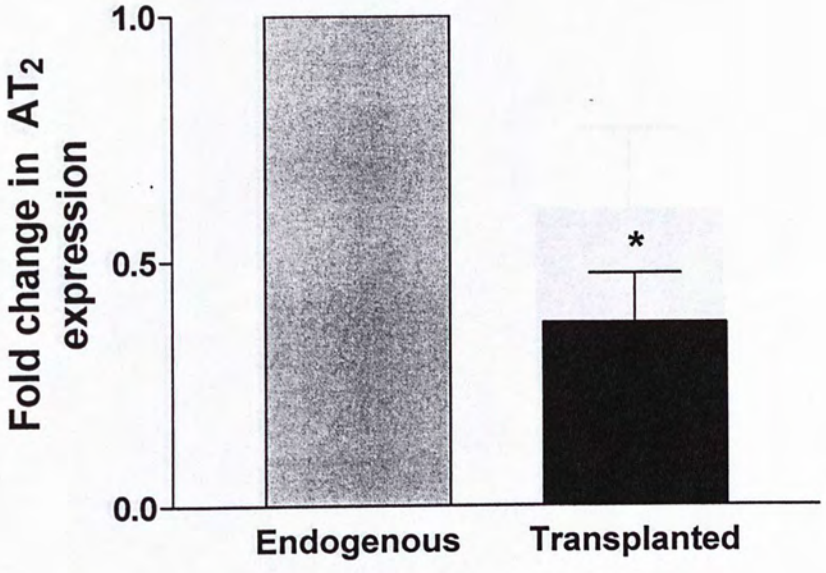


Figure 3-7. Real-time RT-PCR analysis of the mRNA expression of AT_2 receptor in endogenous and transplanted mouse islets. The relative expression was normalized as percentage of β-actin calculated using the comparative C_T method of $2^{-\Delta\Delta C_T}$ (Table 2). All data are expressed as means ± SEM for four experiments in each group. * denotes $P<0.05$ when compared to endogenous islets.

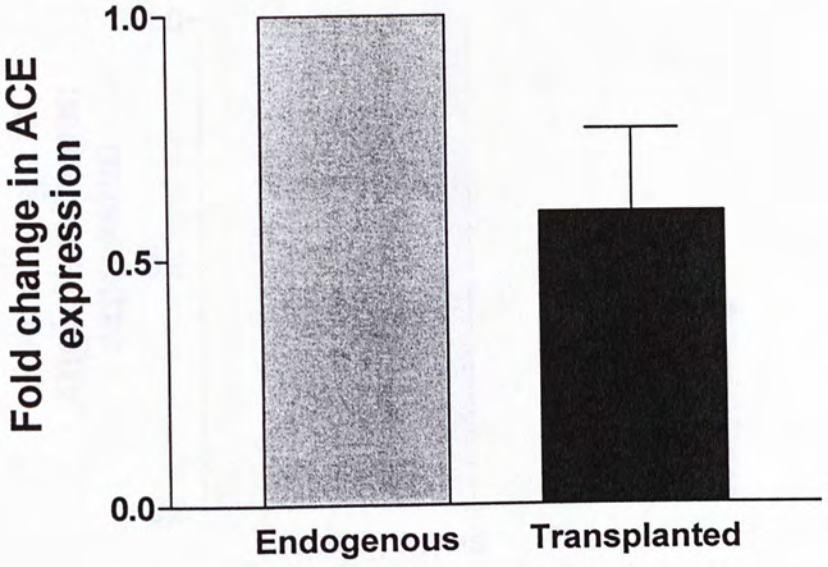


Figure 3-8. Real-time RT-PCR analysis of the mRNA expression of ACE in endogenous and transplanted mouse islets. The relative expression was normalized as percentage of β-actin calculated using the comparative C_T method of $2^{-\Delta\Delta C_T}$ (Table 2). All data are expressed as means ± SEM for four experiments in each group. * denotes $P<0.05$ when compared to endogenous islets.

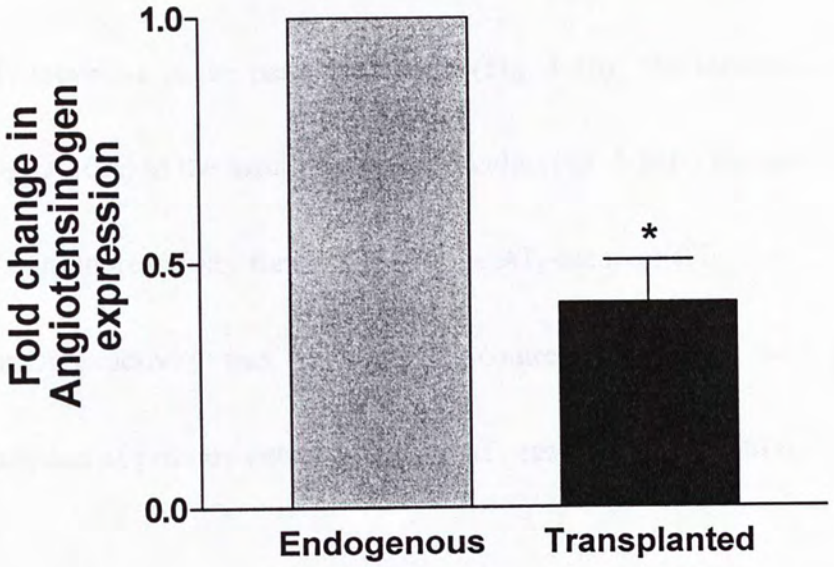


Figure 3-9. Real-time RT-PCR analysis of the mRNA expression of angiotensinogen in endogenous and transplanted mouse islets. The relative expression was normalized as percentage of β-actin calculated using the comparative C_T method of $2^{-\Delta\Delta C_T}$ (Table 2). All data are expressed as means ± SEM for four experiments in each group. * denotes $P<0.05$ when compared to endogenous islets.

3.3.2 Localization of AT_1-receptor in endogenous islets

A technique with double immunostaining was employed for precise localization of AT_1-receptors in the pancreatic islets (Fig. 3-10). The localization of AT_1-receptors (Fig. 3-10A) to the insulin secreting β cells (Fig. 3-10B) was identified by an overlay of immunoreactivity for insulin and the AT_1-receptor (Fig. 3-10C). Specificity of the immunoreactivity was validated by control experiments with preadsorption and omission of primary antibody for the AT_1-receptor (Fig. 3-10D).

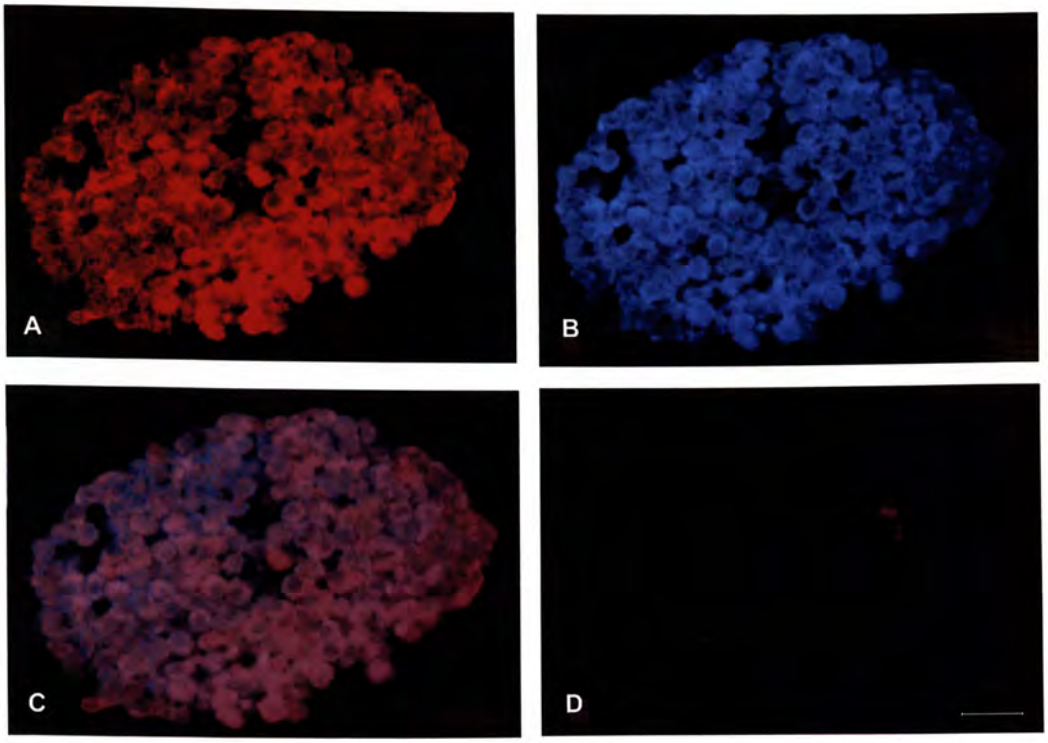
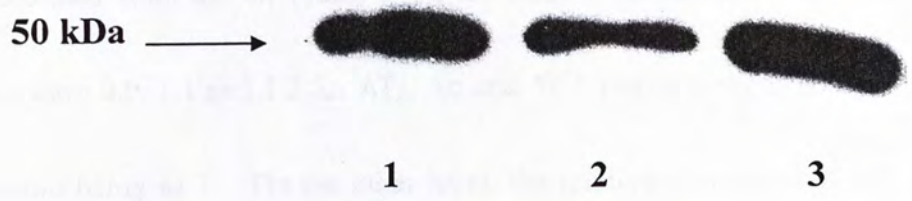


Figure 3-10. Immunohistochemical localization of AT_1 receptors and insulin in isolated mouse pancreatic islets. (A). Isolated islet stained for AT_1 receptors (red). (B). Islet stained for insulin (blue). (C). Immunoreactivity for that AT_1 receptor was co-localized with that for insulin in the islet beta-cells (purple). (D). Negative control with preadsorption and omission of primary antibodies. Magnification: 40 x.
Bar = 40μm.

3.3.3 Expression of AT_1-receptor protein in endogenous and transplanted islets

Western blot analysis was carried out to confirm that findings of AT_1-receptor mRNA in endogenous and transplanted islets were reflected also at the protein level (Fig. 3-11). A major protein band of about 50 kDa was expressed both in endogenous and transplanted islets; in the latter case this was a band of strong intensity (Fig. 3-11A). This molecular mass (50 kDa) was in close agreement with that of AT_1-receptor protein in mouse kidney, which was used as a positive control for the expression of the AT_1-receptor. The relative expression of AT_1-receptor protein in transplanted islets was upregulated two-fold when compared to that in endogenous islets, as determined by image analysis (Fig. 3-11B).

A.



B.

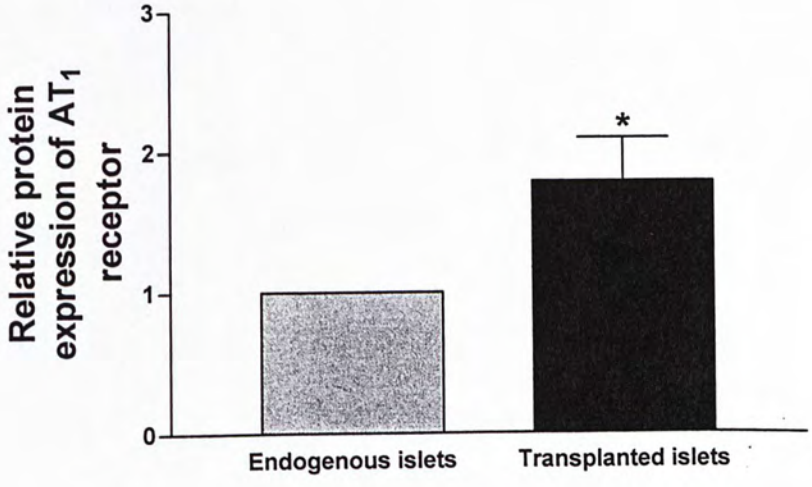


Figure 3-11. Western blot analysis of AT_1 receptor protein from endogenous and transplanted mouse pancreatic islets. A. Representative gel image. Lane 1 shows protein of the AT_1 receptor from mouse kidney. A major band of about 50 kDa was detected. Lanes 2 and 3 show the expression of the AT_1 receptor in protein of 10 μg from isolated endogenous and transplanted islets, respectively. B. The relative expression of the AT_1 receptor protein (%) in transplanted islets when compared to endogenous islets. All data are expressed as means ± SEM for four experiments. * denotes $P<0.05$ when compared to endogenous islets.

3.3.4 Relative abundance of RAS components in kidney and liver

As calculated from the C_T (Table 3-1), the relative abundance of different mRNA studied were 0.9, 1.1 and 1.2 for AT_2, Ao and ACE respectively as compared to AT_1 expression being as 1. On the other hand, the relative abundance of AT_1 and AT_2 expression in the mouse islets was about 70%, when compared with that in the liver and kidney based on the calculation from the C_T using β-actin as internal control (Table 3-2).

Table 3-2. Relative abundance of different RAS components in mouse islet, liver or kidney normalized to β-actin.

Relative abundance (normalized to β-actin)	AT_1	AT_2	A_o	ACE
Islet	0.6193	0.5655	0.6530	0.7625
Liver	0.8465	0.5645	0.8023	0.6480
Kidney	0.8362	0.6218	0.7258	0.8410

3.3.5 Insulin release from perfused transplanted islet graft

A first-phase insulin release in response to stimulation with a high glucose concentration (minutes 15-20) and a sustained second phase (minutes 21-45) was observed from otherwise untreated islet grafts. Addition of Ang II to the high glucose medium during perfusion attenuated the first phase of glucose-stimulated insulin release, and also decreased insulin release during early second phase (Fig. 3-12). When losartan was added to the high glucose medium, a markedly improved early phase of insulin release with a peak value more than twice that in control grafts was observed (Fig. 3-13). In contrast to this, the second phase of insulin release was decreased in these acute losartan-treated grafts. Islet grafts obtained from transplanted animals subjected to losartan treatment throughout the first posttransplantation month had both a lower first and second phase of insulin release compared to control grafts. The insulin content of the islet grafts subjected to chronic losartan was higher than in control grafts (4568$\pm$211 vs 3105$\pm$202 ng insulin/graft, respectively.

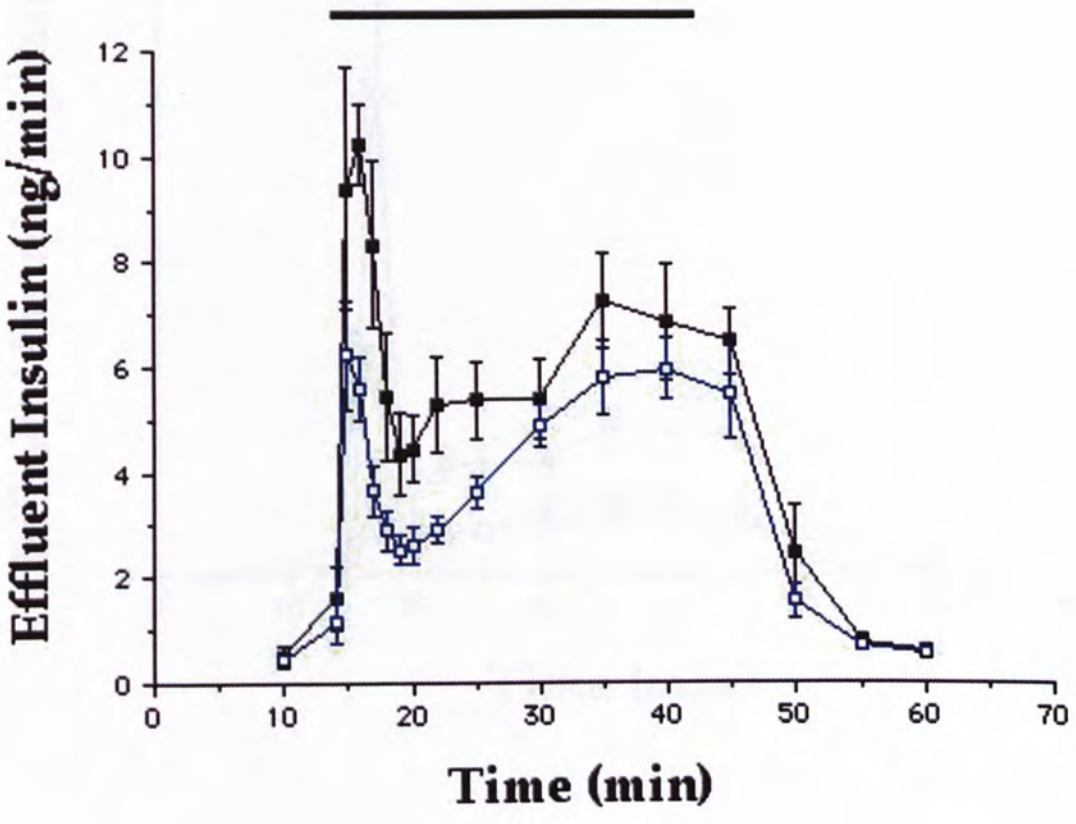


Figure 3-12. Insulin concentration in effluent medium collected from perfused islet graft bearing kidney of C57BL/6 mice. After 15 min of perfusion with a medium containing 2.8 mmol// D-glucose, insulin secretion was stimulated with a 30-min period with 16.7 mmol/l D-glucose (bar), followed by 15 min with 2.8 mmol/l D-glucose. The influence of additon of Ang II (10 nmol/l; empty boxes) to the high glucose medium was compared to the perfusion of control islet grafts (closed boxes). All values are given as means $\pm$ SEM for 6-8 animals. $p<0.05$ for all observations between 15-25 min when compared to control grafts. All comparisons were made using ANOVA.

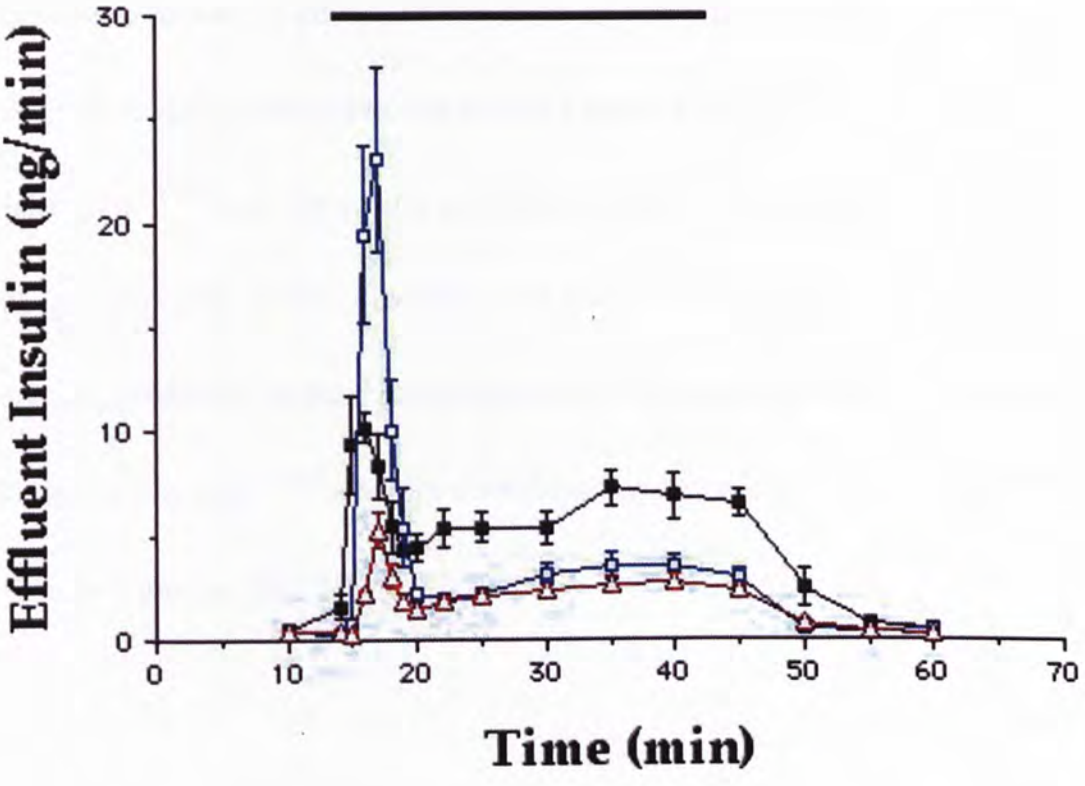


Figure 3-13. Insulin concentration in effluent medium collected from perfused islet graft bearing kidney of C57BL/6 mice. After 15 min of perfusion with a medium containing 2.8 mmol// D-glucose, insulin secretion was stimulated with a 30-min period with 16.7 mmol/l D-glucose (bar), followed by 15 min with 2.8 mmol/l D-glucose.The influence of addition of the Ang II type 1 receptor inhibitor losartan (100µg/ml (empty boxes) to the high glucose medium, or pretreatment of the amimals with losartan (30 mg x kg^{-1} x day^{-1} dissolved in the drinking water; empty triangles) was compared to the perfusion of control islet grafts (closed boxes). Same controls are used in both **3-12** and **3-13**, as well as the different scales on the ordinates. All values are given as means $\pm$ SEM for 6-8 animals. $p<0.05$ for acute losartan treatment at min 16-18; acute losartan treatment at min 20-45 and losartan pretreatment at min 16-50 when compared to control grafts. All comparisons were made using ANOVA.

3.3.6 (Pro)insulin and total protein biosynthesis of transplanted graft

Exposure to losartan 1 μmol/l did not affect (pro)insulin biosynthesis in retrieved islet grafts at high glucose concentration (16.7 mmol/l) (389 ± 108 vs 427 ± 108 dpm x islet graft$^{-1 \times 120}$ min^{-1} for control and losartan exposed grafts, respectively; n=8 in both groups). (Fig. 3-14) Likewise, total protein biosynthesis in the islet grafts were not affected by losartan at the high glucose concentration (803 ± 77 vs 937 ± 231 dpm x islet graft$^{-1 \times 120}$ min^{-1} for control and losartan exposed grafts, respectively; n=7 in both groups) (Fig. 3-15).

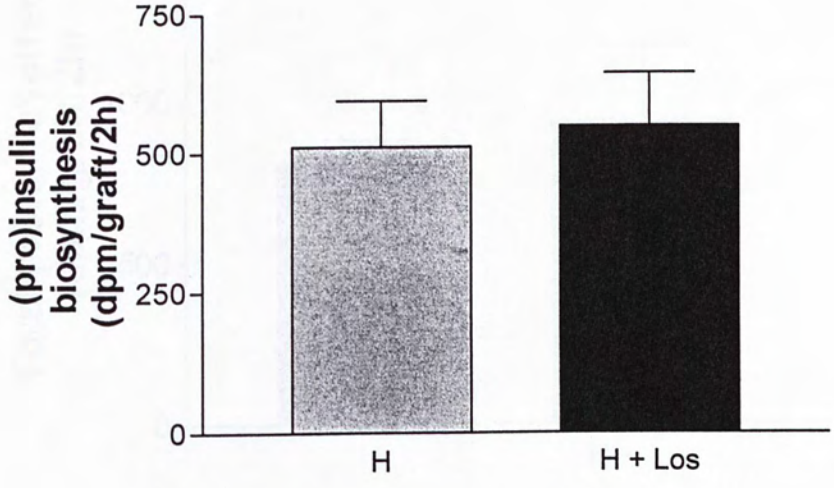


Figure 3-14. Influence of losartan (Los, 1 μmol/l) on the (pro)insulin biosynthesis in islet grafts exposed to 16.7 (high; H) mmol/l glucose. All data are expressed as means±SEM for 7 experiments in each group.

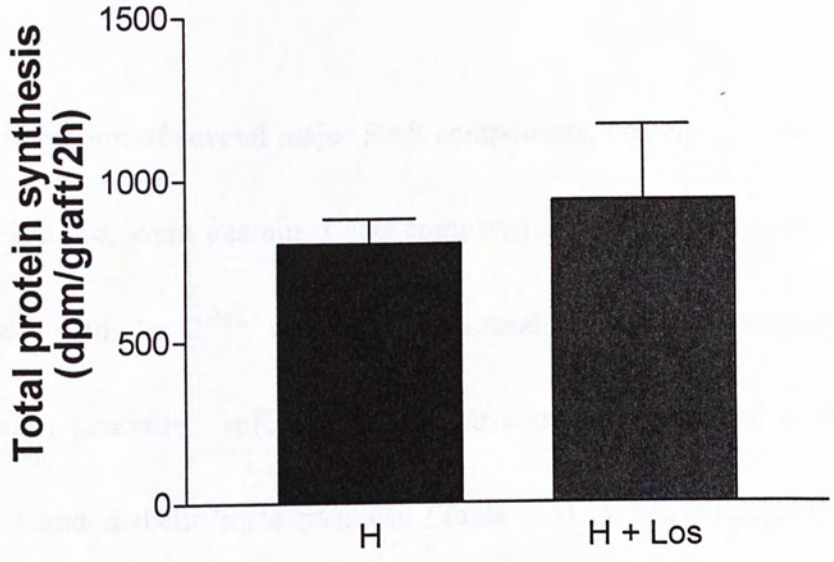


Figure 3-15. Influence of losartan (Los, 1 μmol/l) on the total protein biosynthesis in islet grafts exposed to 16.7 (high; H) mmol/l glucose. All data are expressed as means±SEM for 7 experiments in each group.

3.4 Effects of Ang II and Losartan on islets from diabetic mice

3.4.1 Expression of RAS components in diabetic pancreas

The expression of several major RAS components, namely AT_1- and AT_2-receptors, ACE and Ao, were examined and compared using real-time quantitative RT-PCR coupled with the $2^{-\Delta\Delta C_T}$ comparative method in m+/+db (control) and +db/+db (diabetic) pancreas. mRNAs for all the components studied were expressed in control and diabetic mice pancreas (Table 3-3). When compared to control, the relative expression of mRNAs for AT_1 to β-actin in diabetic mice pancreas was increased by about 2- fold (Fig. 3-16). The relative expression of mRNAs for AT_2 to β-actin was up-regulated by approximately 4.5 fold in diabetic mice pancreas (Fig. 3-17). The expression of mRNA for angiotensinogen mRNA was also increased by almost 7 fold in diabetic mice pancreas (Fig. 3-18), while the expression of ACE in diabetic mice pancreas was down-regulated to about three-quarter of that in control mice pancreas (Fig. 3-19).

Table 3-3. Data on the expression of RAS components in control and diabetic mouse pancreas using the comparative C_T method.

	RAS gene	C_T [a]	ΔC_T [b]	$\Delta\Delta C_T$ [c]	Expression relative to control [d]
Control	*β-actin*	20.19 ±0.67			
	AT1	21.51±0.44	1.51±1.33		
	AT2	35.08±0.97	14.87±0.91		
	ACE	31.77±0.75	11.68±0.69		
	Ao	24.78±0.61	4.83±0.49		
Diabetic	*β-actin*	20.22±1.06			
	AT_1	21.56±0.61	0.65±1.45	-0.87±0.56	1.94
	AT_2	32.84±0.96	12.95±0.99	-1.92±0.69	4.17
	ACE	29.76±0.93	8.99±0.37	-2.69±0.53	6.84
	Ao	25.34±0.88	5.36±0.40	+0.53±0.30	0.70

Ao Angiotensinogen

[a] The average of the C_T data for each sample

[b] The ΔC_T value is calculated by the subtraction of the β-actin C_T from each sample C_T

[c] The$\Delta\Delta C_T$ value is calculated by subtraction of the control ΔC_T from each transplanted sample ΔC_T

[d] The expression relative to control is calculated using the equation $2^{-\Delta\Delta C_T}$

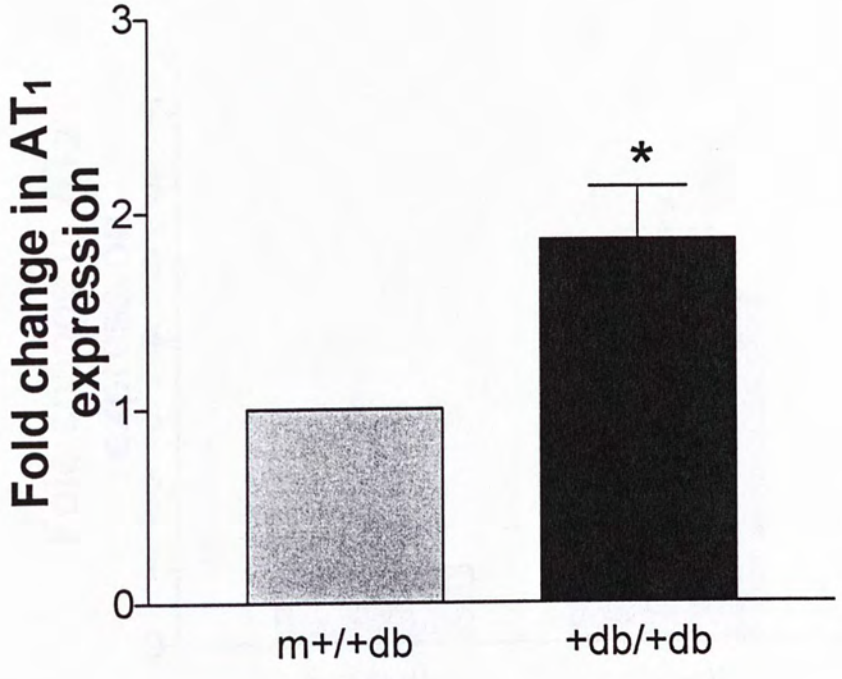


Figure 3-16. Real-time RT-PCR analysis of the mRNA expression of AT_1 receptor in control (m+/+db) and diabetic (+db/+db) mouse pancreas. The relative expression was normalized as percentage of β-actin calculated using the comparative C_T method of $2^{-\Delta\Delta C_T}$. All data are expressed as means ± SEM for six experiments in each group.

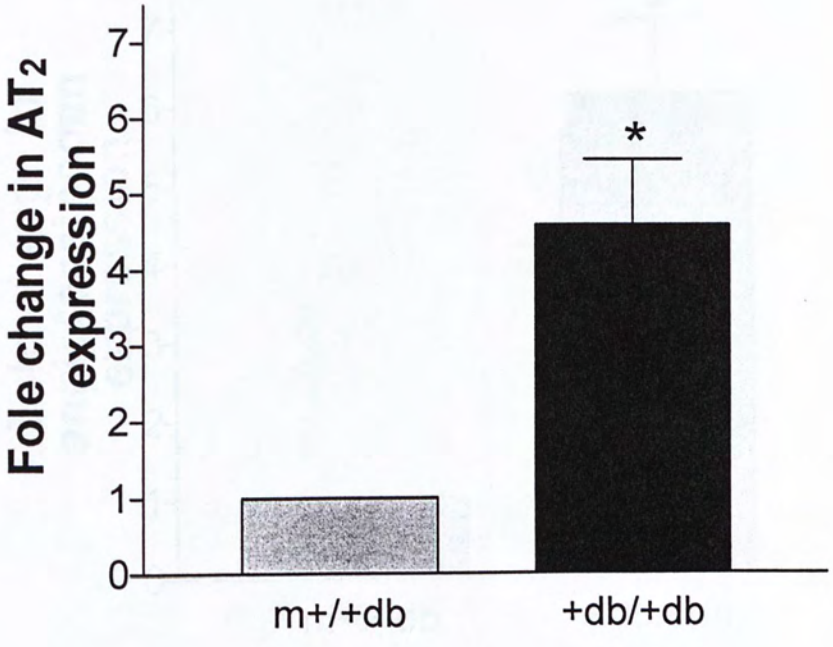


Figure 3-17. Real-time RT-PCR analysis of the mRNA expression of AT_2 receptor in control (m+/+db) and diabetic (+db/+db) mouse pancreas. The relative expression was normalized as percentage of β-actin calculated using the comparative C_T method of $2^{-\Delta\Delta C_T}$. All data are expressed as means ± SEM for six experiments in each group. * denotes $P<0.05$ when compared to control islets.

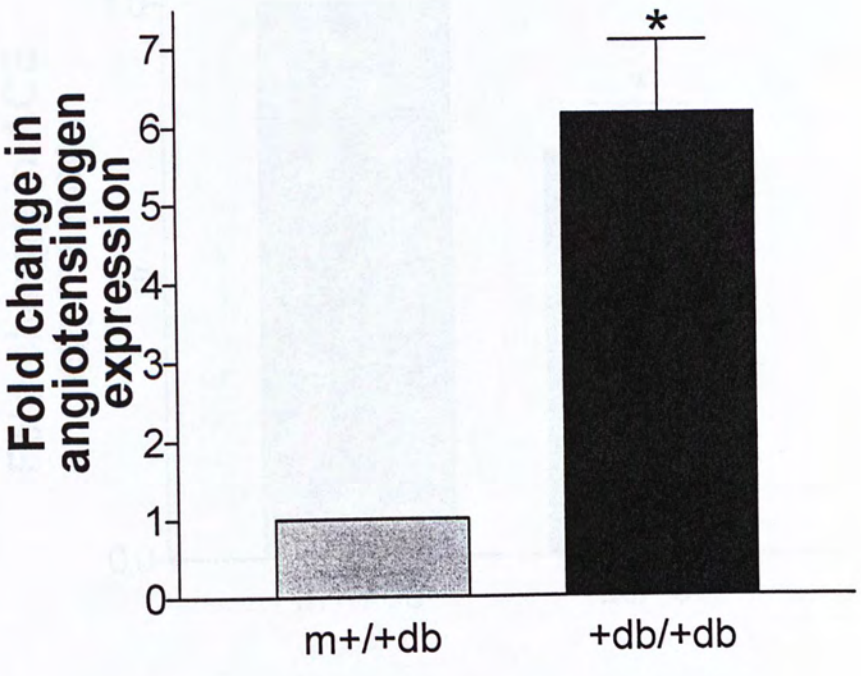


Figure 3-18. Real-time RT-PCR analysis of the mRNA expression of angiotensinogen in control (m+/+db) and diabetic (+db/+db) mouse pancreas. The relative expression was normalized as percentage of β-actin calculated using the comparative C_T method of $2^{-\Delta\Delta C_T}$. All data are expressed as means ± SEM for six experiments in each group. * denotes $P<0.05$ when compared to control islets.

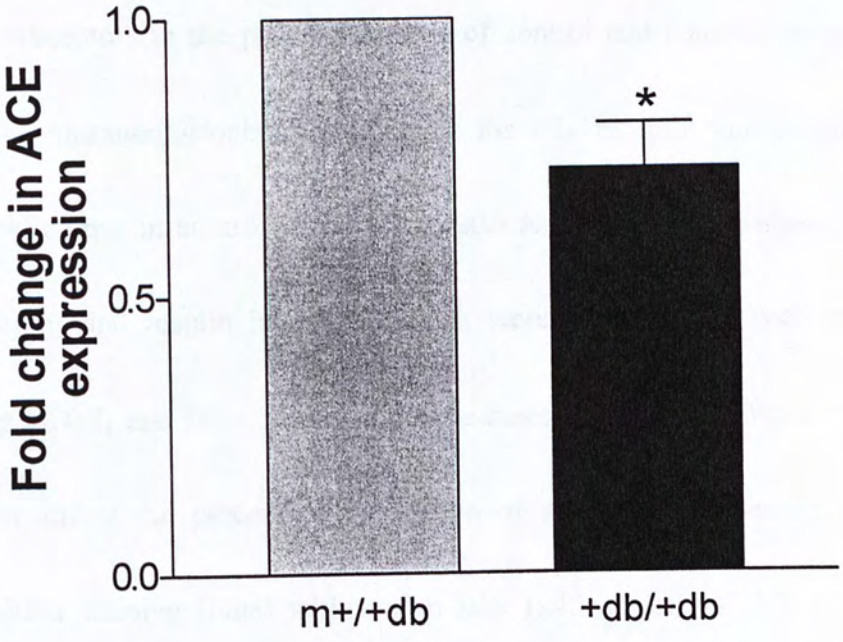


Figure 3-19. Real-time RT-PCR analysis of the mRNA expression of ACE in control (m+/+db) and diabetic (+db/+db) mouse pancreas. The relative expression was normalized as percentage of β-actin calculated using the comparative C_T method of $2^{-\Delta\Delta C_T}$. All data are expressed as means ± SEM for six experiments in each group. * denotes $P<0.05$ when compared to control islets.

3.4.2 Localization of AT_1 receptors in diabetic pancreas

A technique with double immunostaining was employed for precise localization of AT_1-receptors in the pancreatic islets of control and diabetic mouse pancreas (Fig. 3-9). Immunohistochemical staining for AT_1 receptor and β-cell marker insulin were intense in control islets. (Fig.3-20A and B). By comparison, staining for AT_1 receptor and insulin in diabetic islets were diffusing but with stronger intensity. (Fig.3-20 C and D). Semiquantitative assessment of islet β-cell was performed by determining the percentage proportion of area per islet section occupied by the positive staining (blue) within each islet (x40 objective). A total of 10 islets per mouse pancreas were analyzed. There was an overall reduction in the percentage of proportional area positively stained for insulin in diabetic islets (control, 50.60±1.18; diabetic, 24.34±0.77; $p<0.05$)

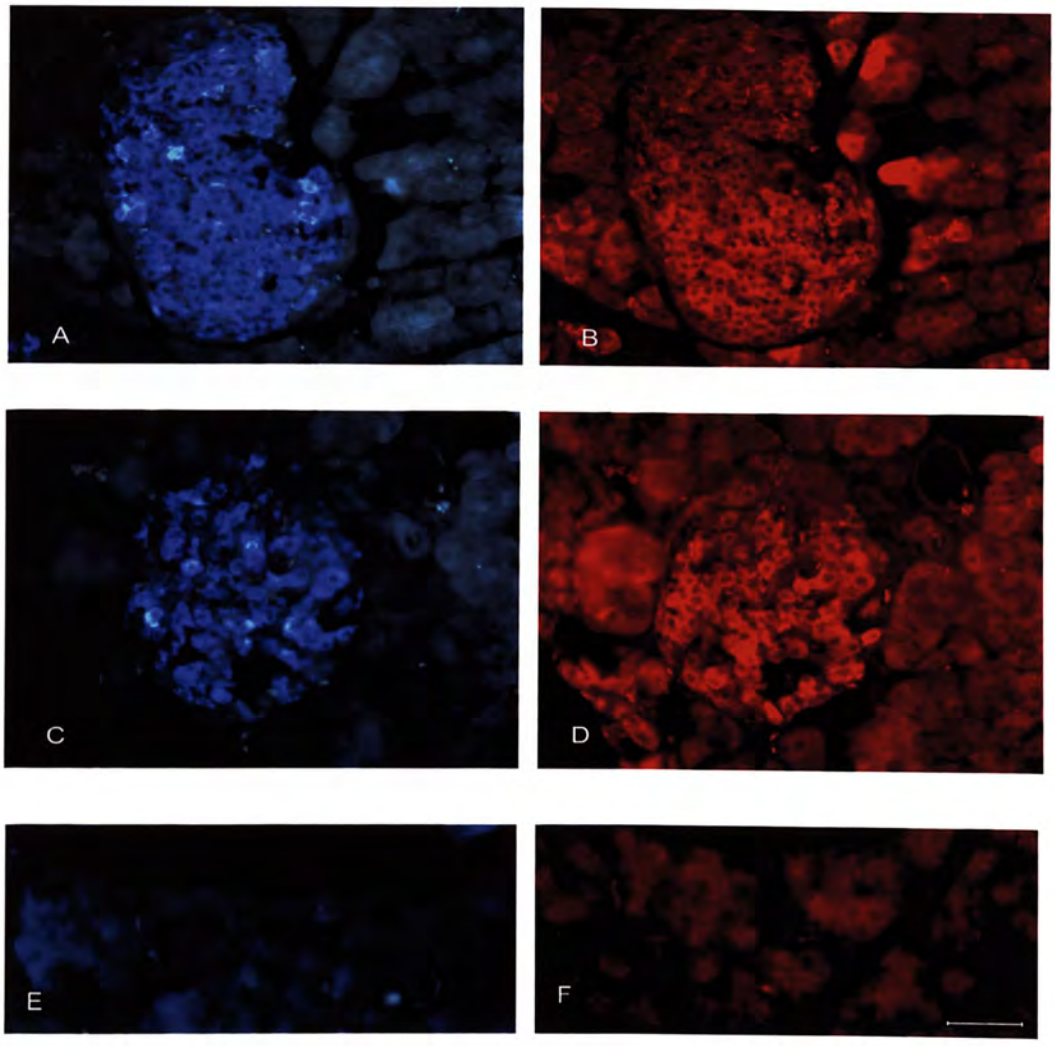


Figure 3-20. Immunohistochemical localization of AT_1 receptors and insulin in mouse pancreatic islets. (A) Pancreatic islet of control (m+/+db) mouse stained for insulin (blue) and (B) stained for AT_1 receptors (red). (C) Pancreatic islet of diabetic (+db/+db) mouse stained for insulin (blue), and (D) stained for AT_1 receptors (red). (E&F) Negative control with the omission of primary antibodies. Magnification: 40 x. Bar = 40μm.

3.4.3 Insulin release from islets of type 2 diabetic mice

For m+/+db (control) mice, insulin release from the isolated islets was markedly enhanced as expected when changing glucose concentration in the incubation medium from 1.7 to 16.7 mmol/l (Fig. 3-21). At the highest used concentration of Ang II (100 nmol/l), the glucose-induced insulin release could be completely prevented. Pretreatment of isolated islets with 1 μmol/l of losartan, a specific antagonist for the AT_1-receptor, before the addition of Ang II (100 nmol/l) completely restored the insulin secretion to the glucose-stimulated level. On the other hand, losartan *per se* had no effect on glucose-stimulated insulin secretion (Fig. 3-21).

For +db/+db (diabetic) mice, insulin release from the isolated islets was also enhanced when changing glucose concentration in the incubation medium from 1.7 to 16.7 mmol/l (Fig. 3-22). At the highest used concentration of Ang II (100 nmol/l), the glucose-induced insulin release could be completely prevented. Pretreatment of isolated islets with 1 μmol/l of losartan, a specific antagonist for the AT_1-receptor, before the addition of Ang II (100 nmol/l) not only completely restored the insulin secretion to the glucose-stimulated level, but also increased insulin release

to a level higher than that with high glucose alone. Losartan *per se* increased the insulin release to a significantly even higher extend (Fig. 3-22).

When combining Fig. 3-21 and Fig. 3-22, it can be seen that the level of insulin release from +db/+db mouse islets is about one fifth of that of control mouse islets.

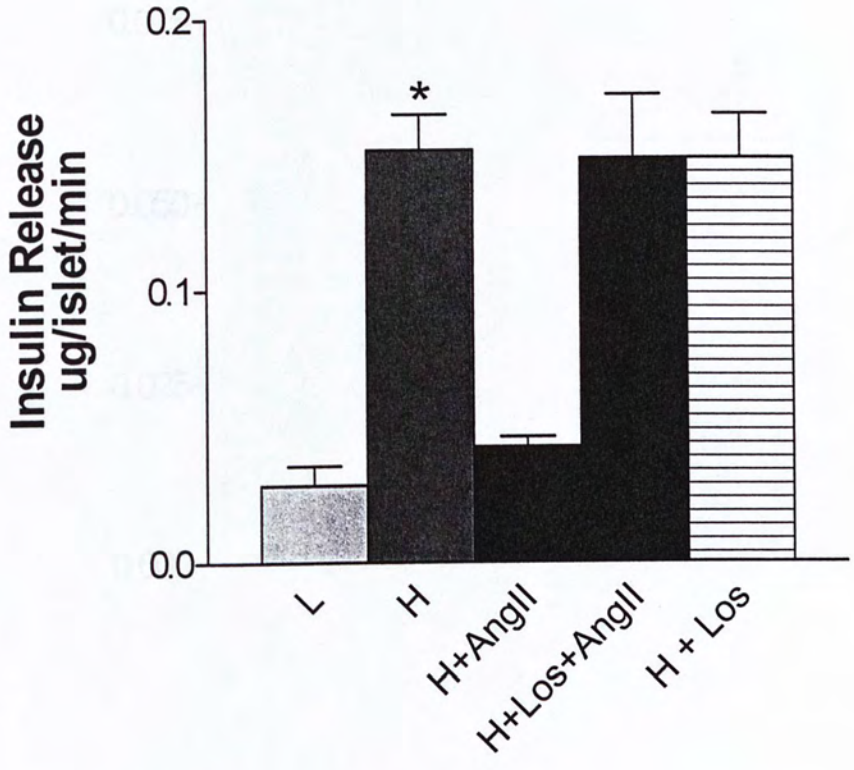


Fig.3-21. Effects of losartan (Los, 1μmol/l) and AngII (100 nmol/l) on insulin release from isolated islets of control mouse (m+/+db) in the presence of 1.7 (low; L) or 16.7 mmol/l (high; H) glucose. *denotes $p<0.05$ when compared to islets exposed to 1.7 mmol/l glucose only.

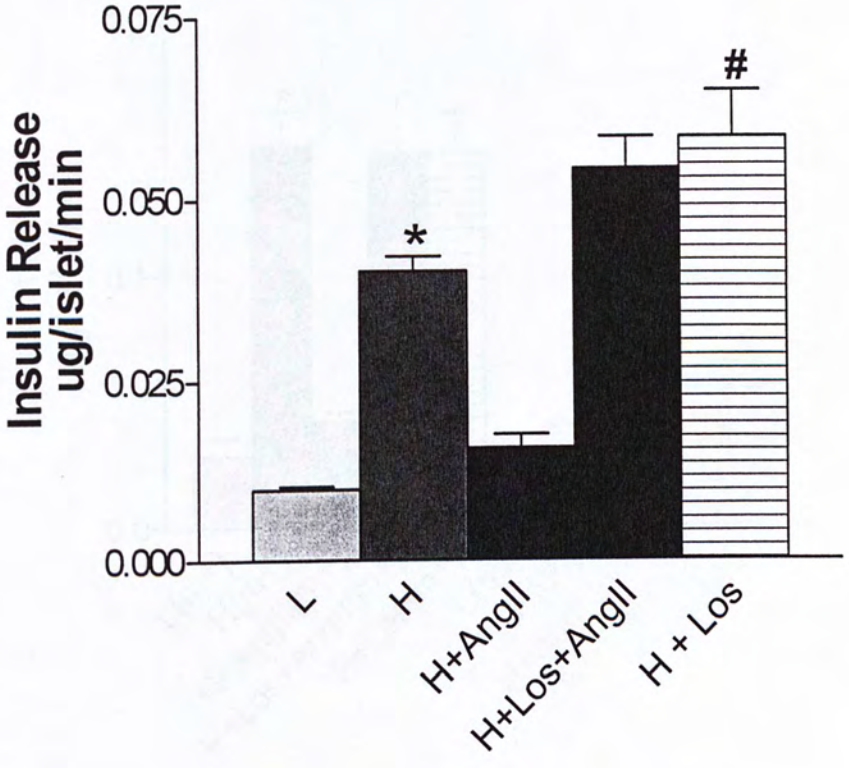


Fig.3-22. Effects of losartan (Los, 1μmol/l) and AngII (100 nmol/l) on insulin release from isolated islets of diabetic mouse in the presence of 1.7 (low; L) or 16.7 mmol/l (high; H) glucose. *denotes $p<0.05$ when compared to islets exposed to 1.7 mmol/l glucose only. #denotes $p<0.05$ when compared to islets exposed to 16.7 mmol/l glucose only.

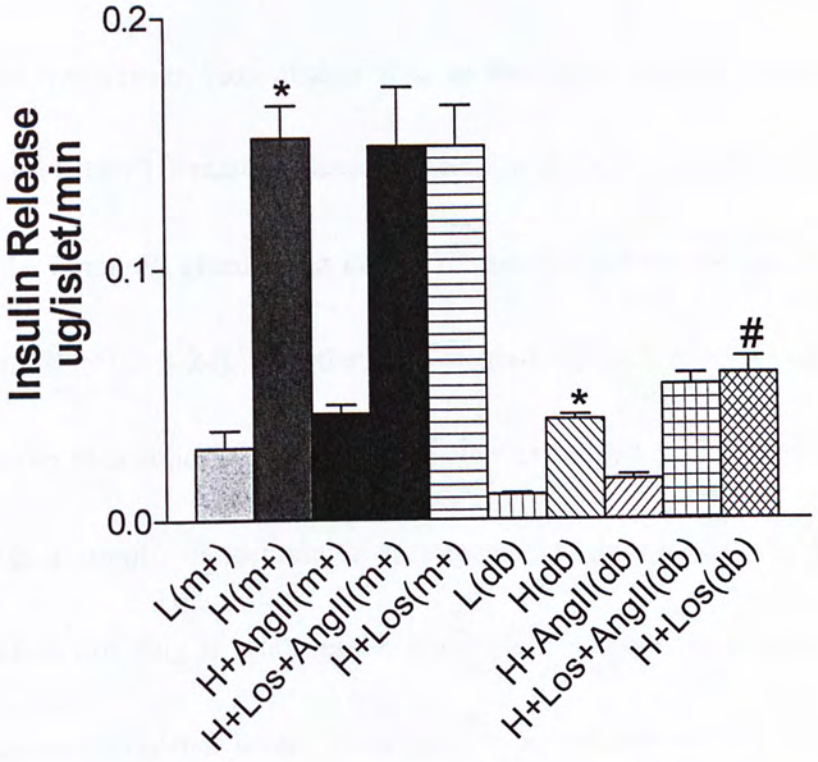


Figure.3-23. Comparison of the effects of losartan (Los, 1 μmol/l) and Ang II (100 nmol/l) on insulin release from control (m+) and diabetic (+db) mouse islets, in the presence of 1.7 (low; L) or 16.7 mmol/l (high; H) glucose.. *denotes $p<0.05$ when compared to islets exposed to 1.7 mmol/l glucose only. #denotes $p<0.05$ when compared to islets exposed to 16.7 mmol/l glucose only.

3.4.4 (Pro)insulin and total protein biosynthesis of islets from type 2 diabetic mice

Islet (pro)insulin biosynthesis was, as the insulin release, markedly higher at 16.7 mmol/l than 1.7 mmol/l glucose (Fig. 3-24 & 3-25). Islet (pro)insulin biosynthesis at 16.7 mmol/l glucose but not at 1.7 mmol/l glucose was inhibited by 100 nmol/l Ang II (Fig. 3-24). At the highest used concentration of Ang II (100 nmol/l), insulin biosynthesis could be completely prevented. Pretreatment of isolated islets with 1 μmol/l of losartan, a specific antagonist for the AT_1-receptor, before the addition of Ang II (100 nmol/l) completely restored the insulin biosynthesis to the glucose-stimulated level. Losartan *per se* increased the insulin biosynthesis to a level higher than that with high glucose alone (Fig. 3-25). By Comparing the effects of losartan on (pro)insulin biosynthesis from control and diabetic mouse islets, it can be seen that glucose-stimulated insulin biosynthesis from diabetic mouse islets was lower than that from control mouse islet. However, addition of Losartan could almost restore the glucose-stimulated insulin biosynthesis to a level similar to that of control islets (Fig. 3-26).

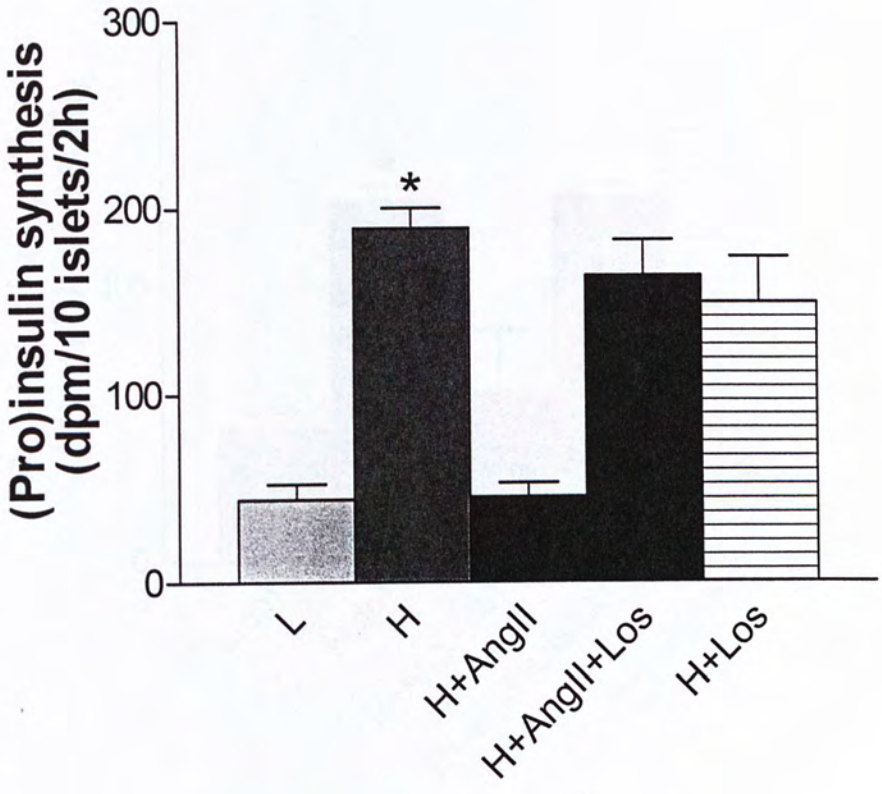


Fig.3-24 Effects of losartan (Los, 1μmol/l) and AngII (100 nmol/l) on (pro)insulin biosynthesis from isolated islets of control mouse (m+/+db) in the presence of 1.7 mmol/l (low; L) or 16.7 mmol/l (high; H) glucose. *denotes $p<0.5$ when compared to islets exposed to 1.7 mmol/l glucose only.

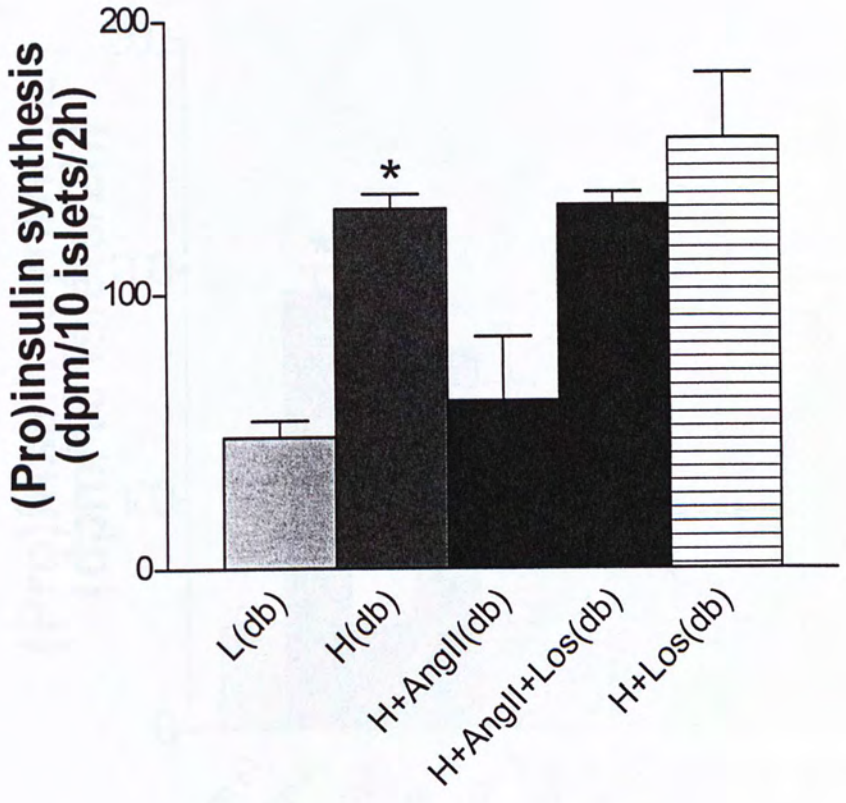


Fig.3-25 Effects of losartan (Los, 1μmol/l) and AngII (100 nmol/l) on (pro)insulin biosynthesis from isolated islets of diabetic mouse (+db/+db), in the presence of 1.7 mmol/l (low; L) or 16.7 mmol/l (high; H) glucose. *denotes $p<0.5$ when compared to islets exposed to 1.7 mmol/l glucose only.

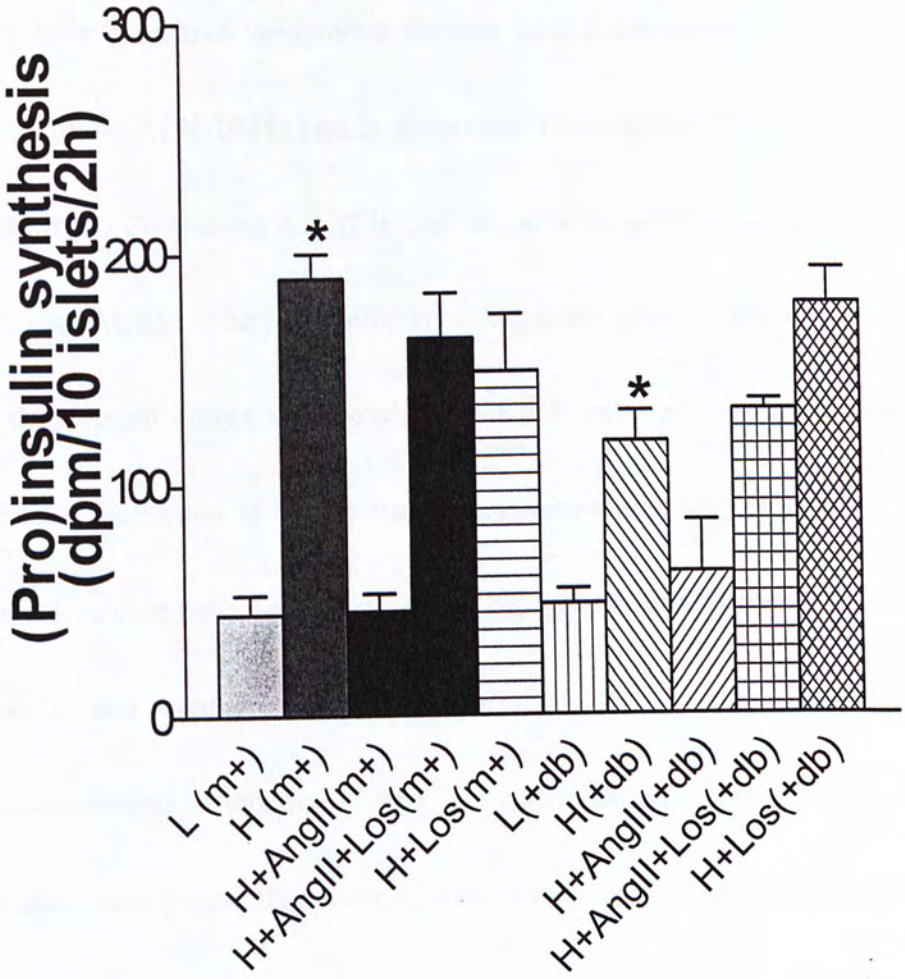


Fig.3-26 Comparison of the effects of losartan (Los, 1 μmol/l) and Ang II (100 nmol/l) on (pro)insulin biosynthesis from control and +db/+db mouse islets, in the presence of 1.7 (low; L) or 16.7 mmol/l (high; H) glucose. *denotes $p<0.5$ when compared to islets exposed to 1.7 mmol/l glucose only.

Chapter 4 Discussion

4.1 Effect of angiotensin II and losartan on islet insulin release

Ang II is the active vasopressor derived from the activation of the RAS. It is an octapeptide (MW 1045) and is generated from its inactive precursor Ang I. The majority of circulating Ang II is derived from the activity of angiotensin-converting enzyme (ACE). The major effects of Ang II are selective stimulation of aldosterone in the adrenal cortex with resultant salt and water retention, vasoconstriction and ultimate promotion of a hypertrophic response within the myocardium and vascular smooth muscle cells, and promotion of cell growth and differentiation within smooth muscle and cardiac cells [Katz 1990]. Studies have shown that a basal intra-pancreatic production of Ang II occurs in the canine pancreas in concentrations several times greater than those measured in peripheral blood [Chappell *et al.* 1991]. Its receptors in the pancreas are found on both endocrine, exocrine and vascular tissue, and are mainly AT_2 receptor and AT_1 receptors [Chappell *et al.* 1992]. Furthermore, a basal intra-pancreatic production of Ang II occurs also in the rat pancreas and this locally produced Ang II can suppress both whole pancreatic and islet blood flow [Carlsson *et al.* 1998].

In that study, islet blood flow changes were associated with changes also in insulin release from the pancreatic islets. The effects of the Ang II on insulin release were made in pancreas with an intact vascular system by measuring insulin concentrations in the effluents from isolated perfused rat pancreata. Results showed that Ang II delayed the first phase of insulin release in response to glucose stimulation [Carlsson *et al.* 1998]. The data concluded that the pancreatic RAS might play a pivotal role in regulating islet blood perfusion, thereby affecting insulin release by the islet cells. As the identity of Ang II receptors on the pancreatic β cells has not been elucidated at that time, vasoconstriction in the pancreatic islets was considered the most likely mechanism for these observations.

To further elucidate the potential effects and mechanism of Ang II on islet insulin release, we employed the technique of islet isolation from the pancreas. We then tested the effects of Ang II coupled with its specific AT_1 receptor antagonist, losartan on islet insulin release in response to glucose stimulation by measuring insulin concentrations in the incubation medium. In the presence study, Ang II induced a dose-dependent and marked decrease of glucose-stimulated insulin release in isolated pancreatic islets, indicating that Ang II plays an important role in direct inhibition of

islet insulin secretion. With the highest dose of Ang II (100 mmol/l) used in the present study, the glucose-stimulated insulin secretion was completely abolished. However, this inhibitory effect could be fully restored by pretreatment of the islets with the specific AT_1-receptor antagonist, losartan (Fig. 3-1 & 3-2).

In contrast to the marked effects of Ang II on insulin release from mouse pancreatic islets as observed in the present study, exposure of the isolated rat islets to Ang II at a similar dose had no effects on insulin release in a previous study [Dunning *et al.* 1984]. The discrepancy of results could be due not only to the species difference but also to the different techniques employed. In this respect, the present study examined the influence of Ang II on glucose-stimulated (16.7 mmol/l glucose) insulin release, whereas the effect of Ang II on insulin release was studied at low glucose concentration (5.6 mmol/l) in previous study [Dunning *et al.* 1984]. The currently employed approach with studies of isolated islets excluded that the inhibitory actions of Ang II on insulin release were solely attributable to its vasoconstrictor actions in the pancreatic islets [Carlsson *et al.* 1998]. The inhibitory effect of Ang II on glucose-stimulated insulin secretion from isolated islets was due, at least partly, to the decrease in (pro)insulin biosynthesis (Fig. 3-4). In

contrast, islet glucose oxidation rate was unaffected by Ang II (Fig. 3-3), indicating that the suppressive action of Ang II on glucose-stimulated insulin secretion appeared not to be of altered glucose oxidation. It has previously been shown that Ang II-receptors could influence prostaglandin synthesis, which in turn may modulate insulin secretion [Jaiswal *et all.* 1984; Kelly & Laychock 1981]. Whether the inhibitory actions of Ang II on insulin release from isolated islets are also mediated by this mechanism remains to be determined.

4.2 Existence of local RAS in pancreatic islets

In recent years, the existence of a local angiotensin-generating system in multiple tissue organs has been demonstrated [Campbell & Habener 1986; Campbell 1987]. This implies that locally produced Ang II exerts local actions from such diverse targets as the heart [Phillips *et al.* 1993], adrenals [Wang *et al.* 1992] and gonads [Leung & Sernia 2003] to the pancreas recently reviewed [Leung & Carlsson 2001]. Previous studies have demonstrated the presence of such a local system in the pancreas of various species, including the dog [Chappell *et al.* 1991], rat [Leung *et al.*

1997; Leung *et al.* 1999], mouse [Leung *et al.* 1998] and human [Tahmasebi *et al.* 1999]. Available data suggest a potential role for the angiotensin-generating system in regulating ductal anion secretion in the exocrine pancreas [Leung & Chappell 2003; Fink *et al.* 2002]. Nevertheless, the potential presence and role of an angiotensin-generating system in the pancreatic islets remains largely unexplored. Indications for a role of Ang II in islets were provided by a perfusion study of whole pancreata, whereby the first phase of glucose-stimulated insulin release was markedly inhibited by Ang II [Carlsson *et al.* 1998]. However, this finding was solely attributable to the vasoconstrictor action of Ang II, which caused a decrease of islet blood flow in the pancreas.

The present study, therefore, aimed specifically to investigate the existence of an angiotensin-generating system in the pancreatic islets. The present study provided evidence for the existence of an angiotensin-generating system in the islets by demonstrating the expression of several components (AT_1 and AT_2 receptors, ACE, and Ao) in the pancreatic islets (Fig. 3-6 to 3-9). Of particular importance in this context is the presence of Ao, which constitutes a mandatory component for a local angiotensin-generating system. The existence of such a system makes a local

production of Ang II probable, which produces its concentrations several times greater than those measured in peripheral blood.

In addition, the AT_1-receptors were localized specifically to the islet β cells as evidenced by double immunofluorescence staining in the present study, further indicating that the inhibitory action of Ang II was mediated through the AT_1-receptor located in the islet β cells (Fig. 3-10). Previous studies have shown that receptors for Ang II have also been identified in the animal pancreatic islets [Leung & Carlsson 2001; Ghiani & Masini 1995], but at least in rat, they were localized preferentially to the surface of α and δ cells [Ghiani & Masini 1995]. In the human pancreas AT_1-receptor positive cells appear, however, to be distributed to those cells stained with insulin [Tahmasebi *et al.* 1999] although the precise localization of the AT_1-receptors has not been unequivocally presented.

4.3 Regulation of islet RAS components by transplantation

Islet cell transplantation provides a possibility to accomplish permanent normoglycemia in patients with type 1 diabetes mellitus [Kenyou *et al.* 1996]. However, its clinical application is severely restricted by the requirement of considerable number of islet cells [Ryan *et al.* 2001; Brendel *et al.* 2001], which might be due, partly, to a lack of proper engraftment thus leading to early graft failure [Jansson & Carlsson 2002]. In this regard, a chronically low oxygen tension and a markedly decreased blood perfusion have been observed in transplanted islets [Carlsson *et al.* 1998, 2000, 2001]. Interestingly, both chronic hypoxia and inflammatory conditions have been shown to activate and upregulate RAS components in the pancreas [Chan *et al.* 2000; Leung *et al.* 2000; Ip *et al.* 2003].

In the present study, the expression of AT_1-receptors, in contrast to the expression of AT_2-receptors, ACE and Ao, was found markedly upregulated both at the mRNA and protein levels in transplanted islets, as demonstrated by real-time RT-PCR and Western blot, respectively (Fig. 3-6 to 3-9, Fig. 3-11). This observation is consistent with the enhanced vascular sensitivity to Ang II, when compared to

endogenous islets, previously recorded in islet transplants [Olsson *et al.* 2000].

As mentioned before, it was observed that exogenous administration of Ang II may inhibit glucose-stimulated insulin release in the first phase through vasoconstrictive effects [Carlsson *et al.* 1998]. The vasoconstrictive effects of Ang II in transplanted islets seemed even more pronounced than in native islets, as also previously observed [Olsson *et al.* 2000]. In the present study, Ang II decreased both the first and second phase of glucose-stimulated insulin release in transplanted islets [Fig. 3-12 & 3-13]. Some of the effects of exogenously administered Ang II, especially on the second phase, may not merely be related to vascular effects, but direct negative effects on (pro)insulin biosynthesis. It is interesting to note that the kinetics of glucose-stimulated insulin secretion from the losartan-exposed islet grafts, with its distinct first peak, much more mimic that of the perfused mouse pancreas than control islet graft without losartan. This may be caused by direct effects of losartan on pancreatic β cells; however it seems less likely in view of the fact that losartan in the present study had no effects on either glucose-stimulated insulin release or (pro)insulin and total protein biosynthesis from retrieved islet grafts (Fig. 3-15). This implicates that the quantities of endogenously produced Ang II in islet grafts, as

in native islets, is not enough to exert negative effects on (pro)insulin biosynthesis. This opens the possibility that the low blood perfusion of transplanted islets normally restricts and delay the output of insulin to the systemic circulation. The lower insulin release in the second phase of losartan-exposed islet transplants would then be explained by a "post-washout" effect. Moreover, the improved first phase of glucose-stimulated insulin exocytosis may also be due to the higher oxygenation of the islet β cells.

The reason for the increased importance of a local RAS in transplanted islets compared to endogenous islets is unclear. However, at least in the exocrine pancreas inflammatory conditions upregulate several components of the local RAS [Leung *et al.* 2000]. High local expression of Ang II may be maintained, or even upregulated by chronically low levels of oxygen tension [Chan *et al* 2000; Ip *et al.* 2002], which seem to occur in islet grafts [Carlsson *et al* 2000]. This hypoxic effect on local Ang II formation may at least partially be mediated through increased expression of vascular endothelial growth factor [Fujiyama *et al.* 2001; Saijonmaa *et al.* 2001].

4.4 Clinical relevance of islet RAS in transplantation

One of the crucial importances for the ultimate function of grafted pancreatic islets or whole pancreas is the revacularization, reinnervation and blood-flow regulation within the transplants. Evidence suggests that the vascular system of transplanted islets is different from that of endogenous islets as characterized by a decrease in vascular density, blood flow and partial pressure of oxygen. Preliminary experiments also suggest that grafted islets have a marked degree of anaerobic metabolism, which might hamper a normal regulation of insulin secretion [Jansson and Carlsson 2002].

Based on the results in the present study, it could be speculated that increased levels of Ang II in islet grafts might support islet revascularization, probably via the effect of up-regulated AT_1 receptors. In view of the low oxygenation of grafted islets after revascularization, the basal vasoconstriction of high local Ang II levels result in a low basal islet graft blood perfusion, and this seems inappropriate in long duration. Though with no well know mechanisms, potential use of AT_1 receptor blocker, losartan, can improve graft blood flow and thus insulin secretion, has shed a light

into optimal conditions for the survival of transplanted islets in clinical trials. This is especially important in view of the shortage of islets which are suitable for transplantation.

4.5 Regulation of islet RAS by Type 2 diabetes

The function of the pancreatic β cell was studied in relation to the development of the diabetic syndrome in a great variety of the diabetic mutant mouse. This was firstly produced at The Jackson Laboratory, Bar Harbor, Maine, U.S.A. by outcrossing of a C57BLl/KsJ-db stock with C57BL/6J mice. The expression of the db-gene in the resulting strain was evaluated by measurements of the body weights and the concentrations of serum glucose and serum insulin at different ages of the animals. In the diabetic mice the body weight was increased rapidly between 5 and 25 weeks of age to a weight twice than the lean controls. During this period, hyperglycaemia and hyperinsulinaemia occur with the maximal serum glucose and insulin values being observed between 17 and 25 weeks of age. Later on, the serum glucose and serum insulin concentrations gradually decrease [Gunnarsson 1975]. This genetic model is used as a model to study the development of defects of insulin secretion in

T2DM. In previous studies, isolated pancreatic islets from male hyperglycemic C57BL/KsJ-db/db mice were used to study (pro)insulin biosynthesis, insulin release and insulin content. A diminution of the insulin content to about 36 % and the insulin release (20 mmol/l glucose) to nearly one half was observed, whereas [3H]leucine incorporation into (pro)insulin was increased. Insulin biosynthesis as well as insulin release showed no further increase, when the glucose concentration was increased to 20 or 30 mmol/l [Wilke and Besch 1982].

In the presence study, we describe, for the first time, the effect of Ang II and specific AT_1 receptor blocker, losartan on isolated islet insulin release in diabetic (db/db) mice. Consistent with previous findings, glucose-stimulated insulin release from db/db mice islets is only about one fifth of that of control (m+/db) mice islets. With Ang II (100 nmol/l), the glucose-stimulated insulin release could be completely prevented. Pretreatment of isolated islets with 1 μmol/l of losartan before the addition of Ang II (100 nmol/l) completely restored the insulin secretion to the glucose-stimulated level, both in diabetic and control islet. In diabetic islets, addition of losartan significantly increased insulin release to a level higher than that with high glucose alone (Fig. 3-22).

As mentioned before, RAS play a pivotal role in regulating islet blood perfusion, thereby affecting insulin release by the islet cells [Carlsson *et al.* 1998]. Addition of Ang II induced a marked vasoconstriction, which could delay the first phase of insulin release in response to glucose. Blockade of the RAS with Ang II antagonist Saralasin induced an increase in whole pancreatic blood flow, notably the microcirculation of islet blood flow. In the presence study, losartan inhibits the vasoconstrictor action of Ang II via the blockade of AT_1 receptor, thus improving intra-islet blood flow and insulin release.

On the other hand, another possible mechanism for the explanation of the improved insulin release by losartan could be due to blockade of amyloid produced in islets of diabetic mice. Amyloid deposits are found in pancreatic islets of 90 % of type 2 diabetic subjects at postmortem. Islet amyloid is formed from islet amyloid polypeptide (IAPP) of the β cells. IAPP is a 37 amino acid peptide which is a normal constituent of β cells and is co-secreted with insulin in animals and in man [Ludvik *et al.*1997]. Previous studies have shown that progressive deposition of IAPP fibrils combined with the associated reduction in the insulin-secreting β cells is likely to contribute to deterioration of islet function in the course of T2DM [Clark *et*

al. 1996 and Wang *et al.* 1999]. In other words, amyloid act as a local inhibitor of stimulated β cells secretion. Evidence has shown that Ang II infusion was associated with increased density of amyloid-binding sites whereas, treatment with ACE inhibitors reduced blood pressure and the density of amyloid binding in the renal cortex [Wookey *et al.* 1998]. Thus, it can be speculated that locally produced amyloid in diabetic mice islet could also be blocked by losartan, thus resulting in improved insulin secretion.

In our study, the local pancreatic RAS components were up-regulated in diabetic mouse model, as evidenced from both real-time PCR and immunocytohistochemistry. AT_1 and AT_2 receptors, and angiotensinogen were up-regulated by this obesity induced diabetes to about 2, 4 and 7 fold, respectively (Fig. 3-16 to Fig. 3-18). In contrast, ACE was down-regulated in diabetic mouse pancreas (Fig. 3-19). The mechanisms for activation of the islet RAS in the diabetic mice have yet to be elucidated.

Angiotensinogen, the substrate from which Ang II is formed, was previously shown to be elevated in adipose tissue of obese (ob/ob and db/db) mice and regulated by

nutritional manipulation such as insulin, glucose, and β-adrenergic agonist. Synthesis of Ang II has been described in adipose cells and has been linked to regulation of adiposity [Jones *et al.* 1997]. Other component of the RAS has also been found in this animal model. ACE and the ACE 2 were measured in kidney and heart from 8-week-old nondiabetic control (m+/db) mice and diabetic (db/db) mice. Results showed that in young db/db mice, ACE 2 protein in renal cortical tubules is increased, whereas ACE protein is decreased. This suggest that the pattern of low ACE protein coupled with increased ACE 2 protein expression may be renoprotective in early stages of diabetes [Ye *et al.* 2004].

Previous studies also showed that chronic hyperglycemia and hyperlipidemia activate the local RAS [Rincon-Choles *et al.* 2002]. Studies of patients with obesity suggest that both weight gain and hypertension activate the RAS [Barton *et al.* 2003; Dal Ponte *et al.* 1998]. In addition, a recent study demonstrated an increased pancreatic expression of RAS components, which is correlated with increased intra-islet fibrosis, apoptosis, and oxidative stress in the Zucker diabetic fatty (ZDF) rats [Tikellis *et al.* 2004].

It is of great importance that pancreatic islets maintain a specialized architecture for

proper function. Disruption of contacts between β cells reduces the secretory efficiency of islets. Loss of cell-to-cell communication associated with increased islet fibrosis may also promote islet cell apoptosis [Ilieva *et al* 1999].

In the diabetic mice model used for the presence study, islets exhibited a reduced proportion of insulin-secreting cells but increased glucagon-, somatostatin, and pancreatic polypeptide -containing cells, as compared with islets of control (m+/+db) mice [Baetens *et al.* 1978]. The changes in cell populations induced a qualitative alteration of cellular interrelationships in the affected islets. Abnormalities in ultrastructures of islet capillaries were also detected in db/db mice, as demonstrated by the visual inspection and morphometry of electron micrographs. The observed changes are: (1) capillary scarcity; (2) increase in the mean and diversity of capillary size; (3) pericapillary edema and fibrosis; (4) hypertrophy of the pericyte and abundance therein of actin-like microfilaments; and (5) luminal irregularity. Changes in (2), (3) and (4) are conceived to indicate hyperperfusion, capillary hypertension and secondary vascular response. In particular, such pericyte changes were found to be shared by other organs whose capillaries are susceptible to diabetic complications [Nakamura *et al.* 1995].

From our immunostaining results, we can see clearly a reduction in insulin producing

β cell number and a disruption of islet architecture. The RAS has been linked to increased fibrosis in various tissues, such as the heart [Seccia *et al.* 2003], kidney [Satoh *et al.* 2001], and liver [Yoshiji *et al.* 2001]. It has been recently known that locally generated Ang II could upregulated the expression of TGF-β, fibronectin, suggesting a role of AT_2 receptor in the regulation of fibrogenic action [Leung *et al.* 2003]

Pancreatic islets are highly susceptible to oxidative injury, because of low endogenous antioxidant activity [Robertson *et al* 2003]. Oxidative stress is produced under diabetic conditions and is likely involved in progression of pancreatic β cell dysfunction as observed in diabetes. Pancreatic β cells are vulnerable to oxidative stress probably caused by low levels of antioxidant enzyme expressions. In β cells, glucose stimulates the production of reactive oxygen species in islet through protein kinase C-dependetn activation of NAD(P)H oxidase [Oliveira *et al.* 2003]. Ang II also increases tissue NAD(P)H oxidase activity [Cai *et al.* 2002]. ACE inhibitors and angiotensin receptor antagonists were shown to indirectly inhibit NAD(P)H oxidase by preventing activation of the AT_1 receptor [Onozato *et al.* 2002]. In the pancreas, recent studies have explored the differential

effects of RAS blockers and their potential use in the treatment of pancreatic inflammation. Prophylactic administration of saralasin, a non-specific Ang II receptor blocker, was shown to improve acute pancreatitis-induced injury in the pancreas, while ramiprilat, and ACEI, did not [Tsang *et al.* 2003]. The protective mechanism of saralasin may be linked to alleviation of the increased oxidative stress caused by upregulation of Ang II receptors during acute pancreatitis [Ip *et al.* 2003]. In addition, prophylactic and therapeutic administration of AT_1 receptor (losartan) and AT_2 receptor (PD123319) antagonists reveals a distinctive action against pancreatitis-induced oxidative stress [Tsang *et al.* 2004]. This beneficial effect may be due, in part, to inhibition of the AT_1 receptor-mediated NADPH oxidase-dependent production of free radicals and the impaired pancreatic microcirculation seen in acute pancreatitis.

Taken together, preservation of islet architecture and inhibition of oxidative stress by RAS blockade could contribute to the improved insulin function.

4.6 Clinical relevance of islet RAS in Type 2 diabetes

Knowledge in diabetes, its treatment and strategies for its prevention make advance daily. Treatment is aimed at keeping blood glucose near normal levels at all times. Training in self-management is integral to the treatment of diabetes. Treatment must be individualized and must address medical, psychosocial, and lifestyle issues

Treatment options for T2DM mellitus currently consist of diet control, exercise, home blood glucose testing, and when dietary treatment and attempts at weight reduction of obesity fail to correct hyperglycaemia, oral hypoglycemic agents are usually prescribed. Insulin thearapy may be required to achieve satisfactory glycemic control even though it is no needed to prevent ketoacidosis in patients with T2DM. Approximately 40 % of people with T2DM require insulin injections.

The major oral medications currently available for treating hyperglycemia in T2DM are the class of compounds known as sulfonylureas, e.g. tolbutamide and glibenclamide, which increase the release of endogenous insulin as well as improve its peripheral effectiveness. A wider choice of preparations has been available, consisting of a second group of compounds, the biguanides, e.g. metformin. These agents reduce blood glucose even in the absence of pancreatic β cell function. A

third class of drugs are competitive inhibitors of intestinal brush-border, called α-glucosidases, which decrease absorption of many carbohydrates from the gut. A representative of this class is acarbose. A fourth class of drugs, the thiazolidinediones, e.g. troglitazone, act as insulin sensitizers.

In recent years, ACE inhibitors and angiotensin receptor blockers provide an alternative for the treatment of T2DM and its complications

Diabetes mellitus is an important cause of nephropathy, end-stage renal disease, and cardiovascular events. Nephropathy occurs in about 40 % of patients with T2DM. It is the leading cause of end stage renal disease (ESRD), and given that treating this condition is a considerable economic burden, the prevention of ESRD is a major public health goal [Rayner 2004]. Ang II has both haemodynamic and non-haemodynamic effects on renal tissue that contribute to the development and progression of nephropathy [Williams 2003]. Increased glomerular pressure associated with diabetes can be compounded by Ang II-mediated constriction of the glomerular arterioles. This in term causes further elevation in microcirculatory pressure within the glomerulus, leading to excretion of albumin, and the development of microalbuminuria and proteinuria [Blantz and Gabbai 1987]. In addition to these

haemodynamic effects, Ang II can cause further glomerular damage via pro-inflammatory effects and pro-fibrotic actions, mediated by factors such as transforming growth factor-b (TGF-β). Ang II also has pro-oxidant and pro-thrombotic effects that further contribute to renal tissue injury [Jaimes *et al.* 1998]. As such, the haemodynamic and non-haemodynamic effects of Ang II support the hypothesis that blockade of the Ang II receptor will limit the progression of nephropathy.

Losartan is the first angiotensin receptor blocker to be licensed by the United States Food and Drug Administration for the treatment of diabetic nephropathy. The current evidence supports the importance of an effective blockade of Ang II receptor activity with losartan for renoprotection in patients with diabetic nephropathy. The landmark RENAAL (Reduction in Endpoints in Non-Insulin-Dependent Diabetes Mellitus with the Ang II Antagonist Losartan) study [Brenner *et al.* 2001] demonstrated that losartan conferred renoprotection and reduced the risk of progression to ESRD in patients with T2DM beyond that afforded by conventional antihypertensive therapy. The results from Losartan Intervention For Endpoint reduction in hypertension study (LIFE) provide additional support for the importance

of Ang II in renal disease [Dahlöf 2002]. The collective experience with losartan suggests that a losartan-based treatment strategy can reduce blood pressure and proteinuria in T2DM associated nephropathy. Furthermore, recent research proved that losartan reduces urinary albumin excretion in normotensive patients with T2DM. In multivariate analysis, the antiproteinuric effect of losartan was independent of the associated reduction in blood pressure and was safe and well tolerated in these normotensive patients.

It is well known that T2DM often occurs together with essential hypertension. Moreover, hypertension is a risk factor for the subsequent development of T2DM [Stern 1995]. It was suggest that some factor(s) common to hypertension and diabetes may underlie the strong association between these diseases. Peripheral insulin resistance is commonly found in patients with essential hypertension and T2DM [Ferrannini *et al.* 1987]. However, it seems that T2DM does not develop as long as the pancreatic β cells can secrete sufficient quantities of insulin to maintain normal glucose homeostasis [Hellerström 1984].

In the Heart Outcomes Prevention Evaluation (HOPE) Study, diabetic patients randomized to ramipril had a 24 % risk reduction compared with diabetic patients randomized to placebo. These data suggest that ramipril, a tissue-specific ACE inhibitor, may provide cardiovascular protection in diabetic patients with associated risk factors even in the absence of a prior history of cardiovascular disease or a diagnosis of hypertension [Yusuf *et al.* 2000]. A decreased diabetes incidence after treatment with an ACE-inhibitor was also noted in the Captopril Prevention Project (CAPP) randomized trial [Hansson *et al.* 1999]. Furthermore, several studies in hypertensive patients receiving long-term treatment with ACE-inhibitors have described an increased initial phase insulin peak in response to glucose administration [Pollare *et al.* 1989; Hänni *et al.* 1994] or oral glucose [Santoro *et al.* 1992]. In this regard, early blockade of the RAS may have a clinically significant protective effect in subjects with impaired glucose tolerance (IGT), a category between normality and diabetes. Subjects with IGT are indeed at increased risk of developing overt diabetes and, even if they do not develop diabetes, atherosclerotic vascular disease [Alberti and Zimmet 1998]. On the other hand, The Nateglinide And Valsartan in Impaired Glucose Tolerance Outcomes Research (NAVIGATOR) trial represents a significant milestone in the quest for prevention of T2DM and

cardiovascular disease. NAVIGATOR is estimated to determine whether long-term administration of nateglinide or valsartan reduce or delay the development of T2DM and cardiovascular disease in people with IGT.

In the present studies with losartan, its beneficial effect in glucose-stimulated insulin secretion seems to be mediated via Ang II receptors directly on β cells in pancreatic islets. These results provide a potential mechanism for the beneficial effects of RAS inhibition in reducing diabetes incidence in at-risk patients with hypertension and other complications, which may have great implications on islet transplantation and T2DM.

4.7 Conclusion

In conclusion, the current study gives evidence for the presence of an islet angiotensin-generating system. This system provides an inhibitory role for locally produced Ang II of glucose-stimulated insulin secretion, an effect mediated by the AT_1 receptors located on the surface of the islet β cells. The increased expression of the AT_1 receptor in islet transplants and in T2DM could have clinical relevance to islet-graft function and T2DM. Application of AT_1 receptor blocker, losartan, could improve insulin secretion in islet graft and in T2DM. On the other hand, perfusion experiments on transplanted islet graft shows a markedly improved first phase of glucose-stimulated insulin release in transplanted islets when exposed to losartan. Inhibition of the local islet RAS maybe, therefore, a feasible strategy to increase the function and improve the structure of the islets in islet transplantation and in T2DM.

4.8 Further studies

Although the pancreatic islet RAS and blockade of this system may have clinical relevance to islet transplant and T2DM, its underlying mechanisms are still not fully understood. Our current results demonstrated the involvement of local RAS in islet transplant and T2DM in isolated islets. Regulation of this system and effects of the RAS blockers should also be studied *in vivo*. Further work on the acute administration and chronic treatment of losartan, with its addition to the post-transplantated and T2DM mice are being undertaken. It will also be intriguing to investigate further the role of the islet RAS in islet graft dysfunction, in view of its adverse effects on islet graft microcirculation and on the β cell mass. To this end, the exact mechanism(s) behind the beneficial effects of RAS blockade on islet transplantation and on reducing the incidence of T2DM, as observed in large clinical trials await further investigations.

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